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Composition and structure of gum Arabic in solution and at oil-water interfaces

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Remerciements

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Overview

Overview

An emulsion consists of a dispersion of a liquid as small droplets (with size ranging from several µm to nm) in another immiscible liquid. Emulsions are present in numerous industrial applications such as food products (milk, butter, mayonnaise or flavored beverages), cosmetic or pharmaceutical products (moisturizing creams, encapsulating active ingredients), bitumen for road coatings or in the paint industry. Such systems are widely used because they combine benefits of both phases. For instance, in a moisturizing cream the aqueous phase brings a fresh sensation while the oil phase contributes to the deposition of a protective film at the skin surface.

Several types of emulsion exist: direct emulsions (oil-in-water), inverse emulsions (water-in-oil) or multiple emulsions (water-in-oil in water for instance). Emulsions are thermodynamically unstable systems. Without the presence of interfacial agents, emulsions break to yield two separate phases. Several types of interfacial agents exist and are used for the stabilization of dispersed systems such as emulsions. A formulation may contain surfactants, nanoparticles, proteins, micro-gels, block-copolymers. All these species are able to adsorb at oil/water interface and to protect droplets against coalescence. The demand for natural additives in formulation is steadily increasing, regarding environmental and health issues. Hydrocolloids constitute a class of natural products such as proteins or polysaccharides, which sometimes possess emulsifying and stabilizing properties. Among these, gum Arabic, an acacia tree exudate, has been used for millennia due to its outstanding interfacial properties. This natural product is a complex mixture of biopolymers. Many attempts have been carried out, with limited success, to mimic interfacial properties of this product. A deeper understanding of the structure and composition of gum Arabic but also of its behavior at oil/water interfaces and of the stabilization mechanisms involved is thus required.

In the first chapter of this thesis, a literature review on the structure, behavior in solution and at interfaces of gum Arabic is presented. In the second chapter, we briefly introduce the analytical techniques used in the frame of this study to improve the understanding of gum Arabic adsorption and stabilization behaviors (emulsification, interfacial tension, chromatographic separation, light scattering and small angle X-ray and neutron scattering).

Chapter two deals with the structure of gum Arabic gum in solution as a multi-component and multi-scale system, which has been investigated using a two dimensional chromatographic separation (size exclusion followed by hydrophobic interaction) and small angle scattering measurements (neutron and X-ray).

Chapter three focuses on the composition of gum Arabic layers adsorbed at oil droplets interfaces within an oil-in-water emulsion, as a function of the physico-chemical parameters (pH and salinity) involved in the formulation, using chromatographic analysis.

In the fourth chapter, the structuration of the gum Arabic adsorbed layer is addressed. We unravel a structuration of adsorbed gum amphiphilic moieties providing an elastic film capable to resist to extreme conditions (such as ultra-centrifugation).

Main results and findings of this work are summarized in the concluding section, and research prospects about the use of gum Arabic in formulation are proposed.

This PhD project was initiated through a collaboration between the **Laboratoire de Génie Chimique** (LGC) and **CARAGUM International®**, through the LabCom **SOpHy**, funded by **ANR**, the French National Research Agency. CARAGUM Int.® (Marseille, France) is a company specialized in the development and sale of food additives, including Gum Arabic.

Chapter I- Literature review

I-1 Introduction

Hydrocolloids are water-soluble polymers which behave in solution more as colloidal particles than as polymers. They usually comprise a wide variety of bio-polymers, typically proteins and polysaccharides that are employed in a broad range of industrial sectors in order to provide specific functions [1]. These functions can include: thickening and gelling aqueous solutions, stabilizing foams, emulsions and dispersions or for the flavour controlled release. Hydrocolloids may originate from different sources: botanical, algal, microbial or animal. These materials are widely used in food industry to influence the texture or the organoleptic properties of the products. Many other industrial products take benefit of their properties such as cosmetics, inks, pharmaceutical or agricultural product [2]–[4].

Starch is the most commonly used product as thickening agent although xanthan gum is more and more employed particularly for its particular rheological properties. Gelatin is widely used as gelling agent. However, since the market demand for non-animal originating product increases, alternatives can be found with carrageenan.

Gum Arabic is a hydrocolloid involved in many industrial processes and especially in the food industry for the emulsification of flavor oil. Because of large fluctuation in the market price of gum Arabic starch-based substitutes to gum Arabic have recently emerged as well as protein-polysaccharides conjugates. The latter are formed through the Maillard reaction or by electrostatic interaction in order to reproduce the properties of gum Arabic. Yet, nowadays, no gum substitute has been found to possess the same exceptional properties as gum Arabic itself, in particular as an emulsion stabilizer. A better understanding of its structure and interfacial properties is still needed in order to mimic and substitute this natural product [4], [5].

The present study focuses on the structural and emulsifying properties of gum Arabic. We present in the following section a literature review on this hydrocolloid, its history, its chemical and structural composition, and finally its properties in solution and as an interfacial agent.

I-2 Literature review on gum Arabic

Gum Arabic is a sticky exudate from the stems and branches of Acacia trees when they are subjected to stress [3], [6]. It is collected as a white to pale amber coloured granular solid as illustrated in Figure I-1. Gum Arabic is named after its geographic location. Acacia trees are indeed present in a wide belt of semi-arid land stretching across sub-Saharan Africa, Sudan being the largest producer [7].

Figure I-1: Pictures of gum Arabic exudates from acacia trees (from Daoub et al., 2016) [8]

Gum Arabic is one of the oldest industrial gums. This natural gum was employed millenniums ago in South and North East Africa for its adhesive properties in tool manufacturing [9]. The ancient Egyptians also used gum Arabic as an adhesive when wrapping mummies and as a pigment binder in mineral paints when making hieroglyphs. Later, gum Arabic has been used as a binder in the formulation of metallo-gallic ink, used by painters between the 12th and the 19th century. Arabic gum has played an important role in the colonization of Africa by European countries, particularly France. Nowadays gum Arabic is still employed for various industrial purposes. Its is used for instance as a flocculating agent in ceramics, as an adhesive in the cosmetic industry, as an additive for micro-encapsulating processes, or for its emulsifying properties in lithography or food products. Recently gum Arabic has also used in the stabilization of individual carbon nanotubes [10]–[13].

I-2-1 Structural composition of gum Arabic

I-2-1-1 Polysaccharide/protein composition

Acacia gum consists of a mixture of polyelectrolytes associated with calcium, magnesium and potassium salts. This hybrid polyelectrolyte contains both proteins and polysaccharides subunits. It is composed of six carbohydrate moieties (galactopyranose, arabinopyranose, arabinofuranose, rhamnopyranose, glucuropyranosyl uronic acid and 4-O methyl glucuropyranosyl uronic acid) and also contains a small proportion of proteins (Table I-1). The main chain is composed of 1,3-linked β-D-galactopyranosyl units. These 1,3-linked β-D-galactopyranosyl units are composed of side chains linked to the main chain by 1,6-linkages. Both the main and side chains contain units of the carbohydrates moieties presented before, the uronic acid moieties being mostly end-units [6], [7], [14], [15].

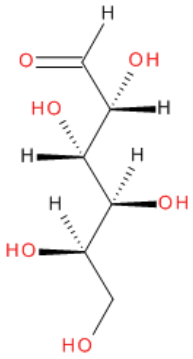	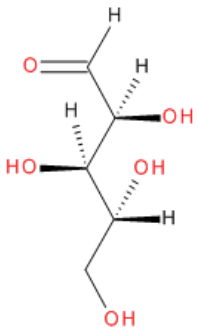	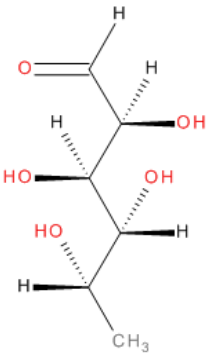	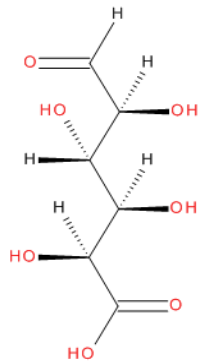
Galatose	**Arabinose**	**Rhamnose**	**Glucuronic Acid**

Table I-1: Main sugar units of gum Arabic

Gum Arabic is approximately composed of 39 to 42% of galactose units, 24 to 27% of arabinose units, 12 to 16% of rhamnose units, 15 and 16 of glucuronic acid units, 1.5 to 2.4% of protein moieties and finally 12 to 16% of moisture. These percentages vary from gums regarding acacia trees ages or locations [6].

The polysaccharide composition will influence gum Arabic solubility (percentage of polysaccharide units compared to that of polypeptide units) and its electrophoretic mobility (mostly influenced by the percentage of glucuronic acid moieties). Moreover, the percentage of protein moieties is a key parameter regarding the adsorption of gum Arabic species at oil/water interfaces.

I-2-1-2 Polydispersity and heterogeneity of gum Arabic species

The chemical composition of gum Arabic does not disclose the distribution of polysaccharide in size or mass and breakdown of proteins in the mixture. Gum Arabic structural composition has been intensively studied since the 1960's. The heterogenous composition of the gum component has been evidenced using electrophoresis [16] or chromatographic techniques [17]. Since then, improved fractionation techniques such as size exclusion chromatography has been used in order to separate gum arabic moieties as a function of their molecular weight [18], [19].

The gum Arabic size exclusion chromatogram (UV detection at 214 nm) obtained by Vandevelde and Fenyo (1985) is presented in Figure 2 Authors showed that the relative mass proportion for each fraction was not related to the area under the curve, indicating a different adsorption behaviour for each gum fraction. Therefore, UV detection was only useful as a qualitative tool.

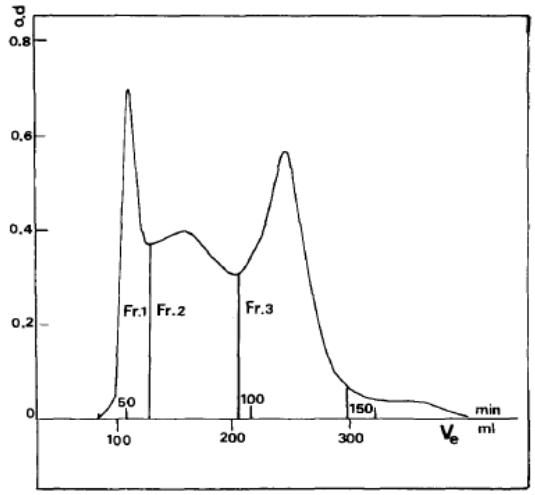

Figure I-2: Size-exclusion chromatography of A.senegal on sephacryl S-500gel in molar sodium chloride. UV detection at 214nm (from Vandevelde and Fenyo, 1985) [19]

The elution profile was shown to be dependent on the origin of the gum sample. However, as a common trend for all gum samples tested, size distributions were always ranging over a broad scale. Three fractions exhibiting differences in viscosity and nitrogen content were identified. The two first contained most of the nitrogen content of the gum and a high viscosity (fract 1 and 2 in Figure 2). The third fraction (fract 3) was mainly composed of low viscosity polysaccharides molecules.

Randall et al. (1988) applied an enzymatic degradation technique on a gum Arabic sample to eliminate the proteinaceous moieties from the gum. A diminution of the intensity of high molecular mass eluted fraction was observed, suggesting that most of the proteinaceous material of the gum was associated to these macromolecules [18].

The relative proportion of each species depends on the geography, soil, variety and age of the trees however the gross chemical and physicochemical characteristics of different gum is quite constant [20], [6], [21]–[23]. Depending on its origin and age, the averaged molar mass of the whole gum can vary from $3.0x10^5$ to $1.0x10^6$ g/mol.

Hydrophobic interaction chromatography was used by the same authors to fractionate gum Arabic [24]. An elution gradient of NaCl concentration going from 4.2 $mol.l^{-1}$ NaCl to zero was applied. Four fractions could be isolated the composition of which is reported in Table I-2. Most of the gum was found to have a low protein content and was referred to as arabinogalactan polysaccharides (AG) (80-90% of the total gum in weight) with an average molecular weight of $3x10^5 g.mol^{-1}$. The minor fractions constituting about 12% of the total mass were rich in proteins and were referred to as: arabinogalactan protein conjugates (AGP) (10% of the total gum in weight) with an average molecular weight of $1.45x10^6 g.mol^{-1}$ and, glycoproteins (GP) (1-2 w/w% of the total gum). The

authors revealed the presence of two possible types of glycoprotein with one of them having an average molecular weight of $2.5x10^5 g.mol^{-1}$.

	Whole gum	Fraction 1	Fraction 2	Fraction 3A	Fraction 3B
% of total recovered[a]		88.4	10.4	1.0	0.24
% galactose	36.2 ± 2.3	34.5 ± 2.2	29.3 ± 0.7	12.3 ± 0.5	–
% arabinose	30.5 ± 3.5	27.6 ± 1.9	31.4 ± 1.0	15.0 ± 1.3	–
% galactose/% arabinose	1.19	1.25	0.93	0.82	–
% rhamnose	13.0 ± 1.1	11.8 ± 2.2	12.9 ± 1.0	6.7 ± 1.1	–
% glucuronic acid	19.5 ± 0.2	23.1 ± 0.4	17.6 ± 0.1	11.2 ± 0.3	–
% protein[b]	2.24 ± 0.15	0.35 ± 0.10	11.8 ± 0.5	47.3 ± 3.0	50.0 ± 5.0
Specific rotation	−30.0	−30.0	−37.5	–	–

All percentages expressed on a dry wt basis.
[a] Yield: >99%.
[b] Calculated using N conversion factor of 6.60 (NB recalculated values determined from actual amino acid contents are given in Table II).

Table I-2: Sugar and protein compositions of A.Senegal and isolated fractions (from Randall et al., 1989) [24]

Relatively small hydrodynamic radii were measured with respect to the high molecular weights, suggesting a highly branched structure. This observation was confirmed by NMR spectroscopy and methylation analysis [24]. Note however that in these experiments, gum Arabic sample was dissolved in a concentrated solution of salt (NaCl at 4.2 $mol.l^{-1}$) and eluted using the same solution. The question of the impact of such a salt concentration on the structure of the native gum species was not addressed.

Sample	Av. mol. mass from GPC data (g/mol)	Av. mol. mass from light scattering (g/mol)	$(\bar{S}^2)^{1/2}/nm$	R_h[a]/nm
Whole gum	–	4.6×10^5	–	14.1 ± 0.5
Fraction 1	3.8×10^5	2.79×10^5	–	9.2 ± 0.5
Fraction 2	1.45×10^6	1.45×10^6[b]	25.5 ± 1.6[b]	22.8 ± 0.5
Fraction 3A	2.5×10^5			

Table I-3: Molecular mass and hydrodynamic radius of gum fraction separated by hydrophobic interaction chromatograhy (from Randall et al., 1989) [24]

Figure I-3 shows a HIC chromatogram of a gum Arabic sample carried out by Renard et al. (2006) using the same protocole as in Randall et al. (1989) .

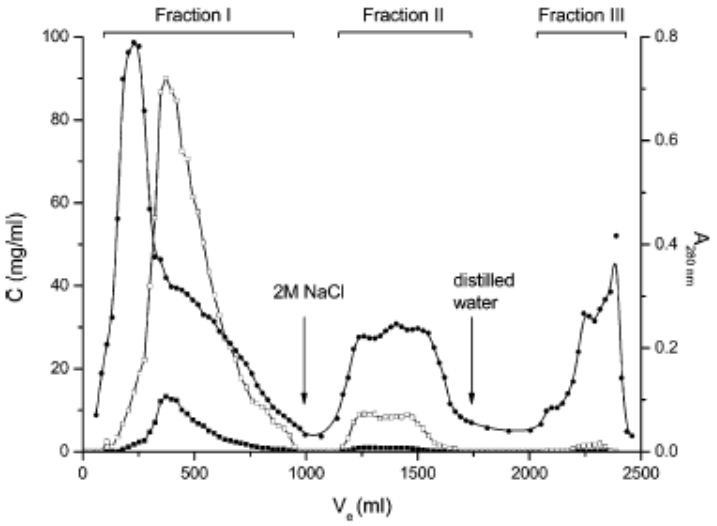

Figure I-3: Elution curve of acacia gum following fractionation by hydophobic interaction chromatography on phenyl-sepharose CL-4B. Molecular fractions were eluted using 4.2M NaCl, 2M NaCl and distilled water. Left traces: neutral sugars (empty square) and uronic acids (black square) concentration C(mg/mL). Right trace: absorbance at 280nm (black circle) (from Renard et al., 2006) [25]

The proteinaceous component of the bulk and the conjugate fractions were found to include similar amino acid distributions, with hydroxyproline and serine being the most abundant (Table 4). However the amino acid composition of the third fraction was different, with aspartic acid being the major amino acid [24], [26]. However studies based on 1H, 13C NMR spectroscopy and methylation analysis showed that there were no main differences between the sugar compositions of each fraction [15].

	Whole gum	Fraction 1	Fraction 2	Fraction 3A
Hydroxyproline	41.0	5.8	291.0	228.0
Aspartic acid	12.0[a]	2.1	30.0	432.0
Threonine	12.0	2.6	67.0	168.0
Serine	22.0	5.4	115.0	324.0
Glutamic acid	6.2	3.1	7.2	324.0
Proline	13.0	2.9	55.0	216.0
Glycine	9.3	2.7	27.0	312.0
Alanine	4.9	1.9	7.8	192.0
Cystine		0.4		11.9
Valine	5.9	1.4	8.7	300.0
Methionine	0.3	0.08		6.0
Isoleucine	2.0	0.7	2.6	84.0
Leucine	12.0	2.0	51.0	300.0
Tyrosine	2.8[b]	0.42	4.6	83.0
Phenylalanine	6.0[b]	0.37	10.0	372.0
Histidine	8.8	1.2	51.0	132.0
Lysine	4.3	0.8	9.9	168.0
Arginine	1.5	2.2	1.2	47.0
% protein[c]	2.03	0.44	9.18	46.7
Protein expressed as a % of the total protein in the whole gum	100.0	20.1	49.5	24.2

Values quoted in nmol/mg gum (dry wt).
[a] An unknown component co-eluting.
[b] Some glucosamine included.
[c] % protein by wt calculated by summing the amino acids recovered.

Table I-4: Amino acid composition of A.Senegal and isolated fractions (from Randall et al., 1989) [24]

The addition of Yariv reagent in gum Arabic solutions precipitates arabinogalactan-protein conjugates. Osman et al. observed that all fractions precipitated clearly indicating the presence of conjugates in each fraction [27].

Several studies have dealt with the two-dimensional separation of gum Arabic macromolecular species. Fractions recovered from hydrophobic interaction chromatography are further separated by size exclusion chromatography [24], [25], [27]. Results from Randall et al. (1989) are displayed in Figure I-4. The less hydrophobic fraction (fraction 1 in Figure I-4 legend) was mainly composed of intermediate molecular mass species. Intermediate hydrophobicity fraction (fraction 2) was eluted first in SEC, and was thus composed of the higher molecular mass. Finally the most hydrophobic fraction (fraction 3A) was eluted last, indicating a composition of smaller molecular mass. However, all recovered fractions were distributed in molecular mass.

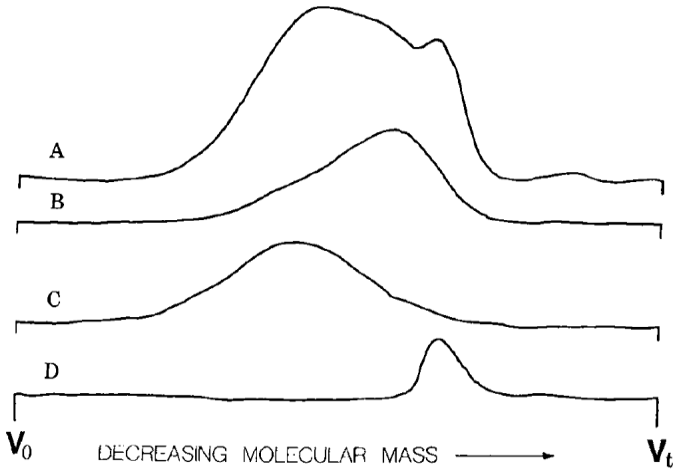

Figure I-4: Size exclusion chromatograms of gum arabic and its fractions (hydrophobic interaction chromatography): (A) whole gum arabic; (B) fraction 1; (C) fraction 2; (D) fraction 3A (from Randall et al., 1989) [24]

Results from Osman et al. (1993b) also indicated that each fraction (of different hydrophobicity) was quite polydisperse in molecular mass. Indeed each fraction once again contained a broad range of molecular mass at different ratios. This result illustrated the hetero-poly-molecular nature of the gum.

Conclusions of Renard et al. (2006) were slightly different from those of Osman et al. (193b) and closer to those of R.C. Randall et al. (1989). In their study, the first fraction recovered from hydrophobic interaction separation was found to be rather monodisperse, contrasting with the other

fractions and especially the third fraction (higher hydrophobicity) spreading over the entire range of molecular mass as shown in Figure I-5.

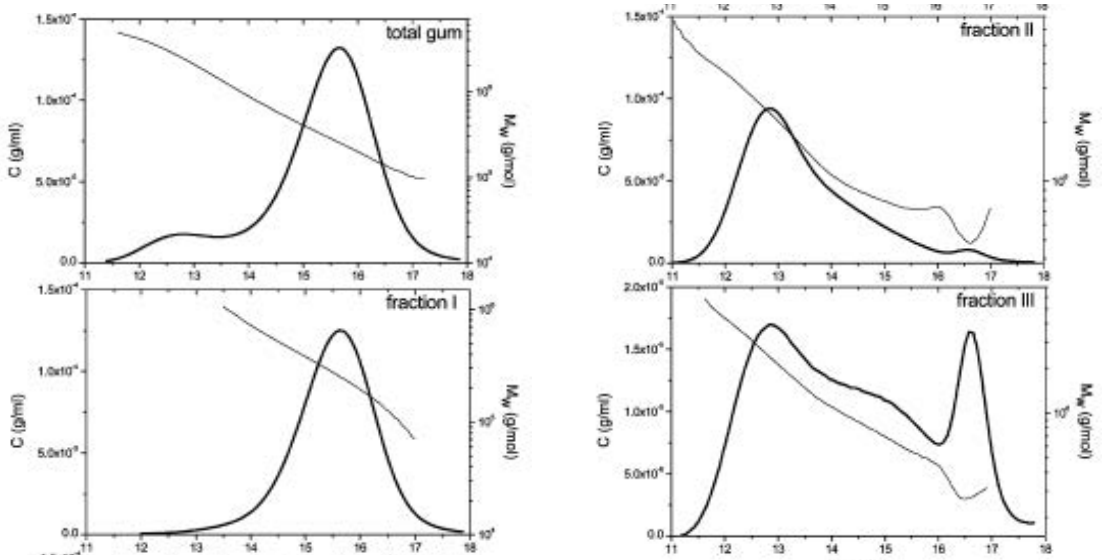

Figure I-5: HPSEC chromatograms showing the elution profiles monitored by refractive index (RI) (C, g/mL) and light scattering (LS) (Mw, g/mol) for acacia gum and its molecular fractions collected after hydrophobic interaction chromatography: RI (tick line), LS (thin line) (from Randall et al., 1989) [25]

The composition of gum issued from old acacia trees contains higher fractions of conjugates compared to that of the gum collected from younger trees. Such an evolution is also observed with aging gum after harvests. A process of gum Arabic maturation has been developed in order to increase the gum emulsifying activity and viscosity [28], [29]. The treatment is performed under strictly controlled temperature and humidity conditions and no chemical reagent is added. This maturation process was described by the authors as similar to the natural development of the gum.

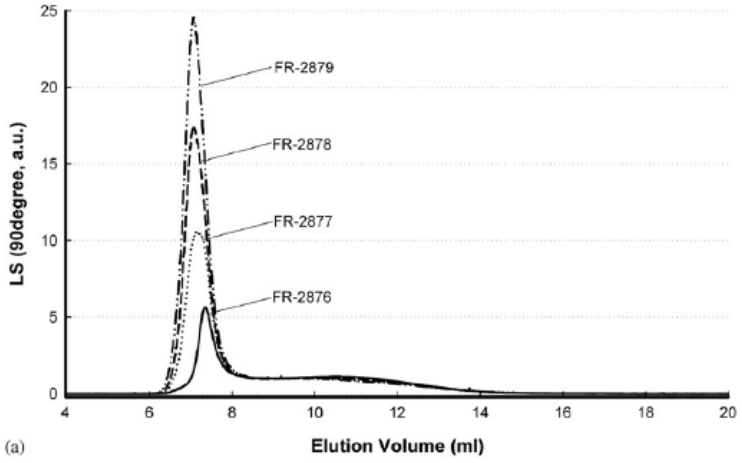

Figure I-6: SEC chromatogram of control (FR-2876) and matured gum arabic. Light scattering detection at 90° (from Aoki et al., 2007a) [29]

Different matured gums were analyzed by size exclusion chromatography and it was shown that the maturation process was increasing the percentage of higher molecular weight molecules within the gum, from 2.5 x10^6 g/mol for the native gum to 11.6 x10^6 g/mol for the mature gum (Figure I-6). Such a process thus accelerates the biological process by associating glycoproteins with free arabinogalactan polysaccharides. Viscosity measurements indicated a highly branched structure. The authors observed no major changes in the gum species chemical structure after maturation [30].

All these studies on gum Arabic fractionation firstly revealed its heterogeneous composition in molecular mass. According to hydrophobic interaction chromatographic separation, the gum has been identified as a mixture of three fractions (arabinogalactan polysaccharides, arabinogalactan-protein conjugates and glycoproteins), which differ in their molecular mass and protein rate. Addition of Yariv reagent has shown that all species from the gum spectrum were present in the form of protein-polysaccharide conjugates. The hydrodynamic radius measured suggested that these macromolecules possess a hyper-branched structure. Finally, studies using two-dimensional separation of gum moieties have shown that the hydrophobicity of each fraction was more or less associated to a molecular mass, with the glycoprotein fraction exhibiting a broad range of molar masses.

I-2-1-3 Structural model of gum Arabic conjugates

Enzyme degradation experiments on gum Arabic samples were conducted by Connally et al. using a proteolytic enzyme, the pronase [31]. The pronase hydrolyses the protein, leading to the release of homogeneous polysaccharides chains of molecular mass similar to that of the arabinogalactan polysaccharides, making up the bulk of the gum. AGP complexes could be identified by a “wattle blossom” model, where carbohydrate blocks are covalently linked to a central polypeptide chain [24], [31]. It was proposed that each AGP molecule consists in average of five carbohydrate blocks of molecular weight around 2.10^5 g.mol^{-1}, linked together by amino acid residues to form a very compact structure [24]. A schematic illustration is given in Figure I-7 and shows the conjugate resembles a block copolymer, which would explain its ability to stabilize emulsions by strong steric repulsion.

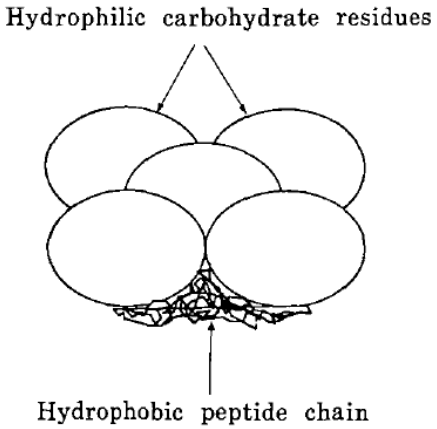

Figure I-7: Wattle blossom proposed model for the structure of arabinogalactan-protein conjugates (from Randall et al., 1988) [18]

An alternative to the "wattle blossom" model was proposed by Qi et al. [32]. Thanks to hydrogen fluoride deglycosylation of the high gum molecular weight fraction (recovered via size exclusion chromatography) there were able to recover a hydroxyproline-rich protein backbone (~400 amino acid residues). Further hydrolysis of the conjugates displayed that most of the carbohydrate groups were attached to the protein through hydroxyproline-galactose linkages. Further analysis of the conjugates structure (chromatographic analysis and TEM) led the authors to name it "twisted hairy rope" structure, schematized in Figure I-8. It consists of a rod-like molecule with small carbohydrate units attached at regular intervals to a polypeptide backbone.

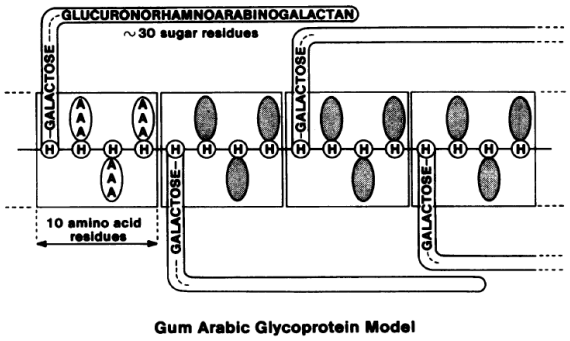

Figure I-8: Hypothetical statistical model of the GAGP. A hypothetical repetitive block size of 7kD contains 10 amino acid residues: 1kD; 30 sugar residues: 4.44KD; 3 hyp-triarabinosides: 1.32kD. The glucuronoarabinogalactan is probably a galactan backbone with glucuronic acid, rhamnose, and arabinose side branches (from Qi et al., 1991) [32]

This model was further amended by Goodrum et al. (2000) with a more symmetric structure as shown in Figure I-9, possibly the source of some properties of the gum. It was suggested that symmetry in peptide moieties may enhance molecular packing and self-association [33].

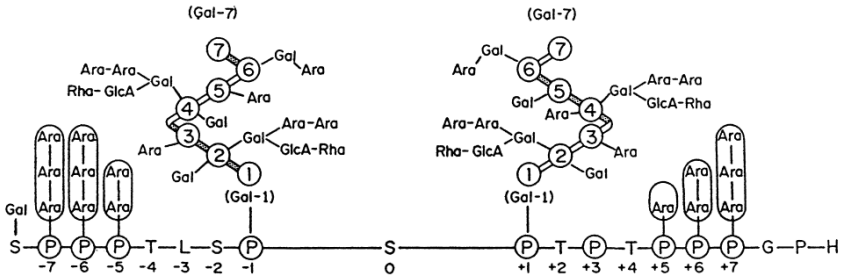

Figure I-9: Model of the GAGP consensus glycopetide according to L.J. Goodrum et al. This model depicts a symmetrical distribution of arabinosides and polysaccharide substituents which is direct by the palindrome-like arrangement of the Hyp residues in the peptide backbone; Ser-O is the palindromic center (from Goodrum et al., 2000) [33]

An improved structure was proposed by Mahendran et al., illustrated in Figure I-10 [34]. Carbohydrate blocks are linked to the protein chain mainly by its serine amino acids and some linkages occurs with the hydroxyproline moieties. This model is consistent with the previous proposed models of wattle blossom (Connolly et al. 1987) and twisted hairy rope (Qi et al. 1991), with a modification in the molecular weight of the carbohydrate moieties attached along the polypeptide backbone. The authors obtained rather different polypeptide length through deglycosylation, with two chains of 45 and 250 amino acids. They also performed both enzymatic and reducing attacks of gum Arabic and claimed that polysaccharides blocks were much smaller than what was reported in enzymatic degradation studies. However, their conclusion does not actually seem to match their experimental data. Rather it seems that a soft reducing attack on arabinogalactan-protein, which mainly cuts O-serine bonds, gives both the polysaccharide blocks and the polypeptide, whereas enzymatic attacks cut the polypeptide. This scenario is consistent with the extensive enzymatic degradation study of Renard et al. [35] on their HIC fraction II, arabino-galactan protein conjugates. They show that this degradation yields similar sizes and structures are the population of arabinogalactan-peptides. The polypeptide chain is attacked with various degrees depending on the enzyme specificity, which can either enhance or degrade secondary structures observed using circular dichroism.

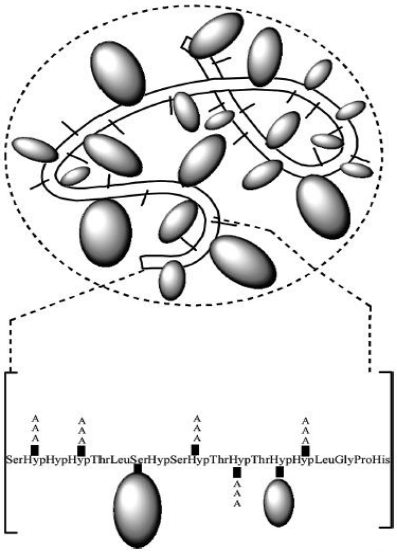

Figure I-10: Schematic illustration of the structure of the gum arabic arabinogalactan protein conjugate. The molecule has a molecular mass of (1-2).10^6 Da and consists of a polypeptide chain possibly containing ~250 amino acids with short arabinose side chains and much larger blocks of carbohydrate of molecular mass ~4.10^4Da and attached. The carbohydrate is highly branched and may adopt the thin oblate ellipsoid structure proposed for the AG component. The amino acid sequence for the polypeptide chain has been determined by Goodrum et al. The molecule adopts a very compact conformation with Rg of ~36nm (from Mahendran et al., 2008) [34]

I-2-1-4 Structural model of gum Arabic in solution

The structure of gum Arabic or gum Arabic fractions has been analyzed by two groups using Small Angle Neutron of X-ray Scattering techniques (resp. SANS and SAXS). Dror et al. have investigated the conformation of Arabic gum in solution at different concentration [36]. Above a concentration of 0.5 wt% a correlation peak was observed in the low-*q* range (large distance). This peak is characteristic of assemblies of scattering moieties with a given inter-aggregates correlation length. At the higher concentration studied (10-30 w/w%) the peak becomes broader as observed in Figure I-11. This result was interpreted as an overlapping of polymer aggregates, in agreement with rheology measurements in aqueous solutions of the gum. The same measurements were conducted with increasing concentrations of salt. It was shown that above a certain concentration (0.5 mol.L^{-1} NaCl) the correlation peak disappeared, indicating that electrostatic interactions determine the distance between the gum aggregates.

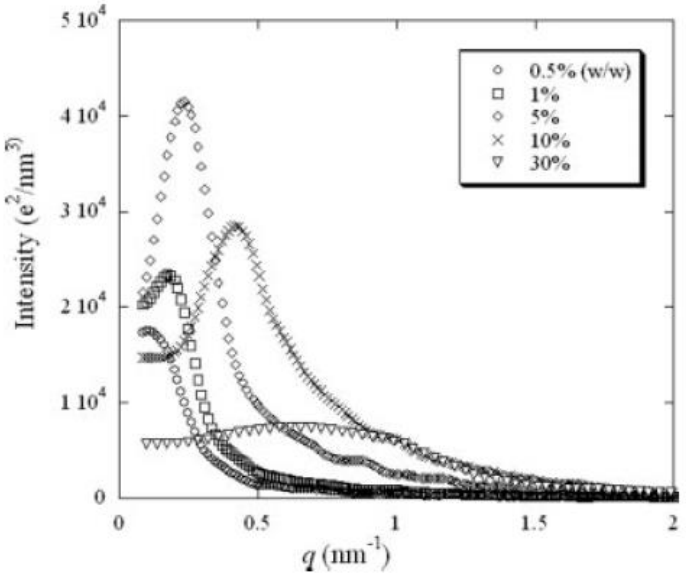

Figure I-11: SAXS spectra of gum arabic solutions at different concentrations (from Dror et al., 2006) [36]

Moreover, these scattering experiments revealed that the gum Arabic is possibly composed of many spheroidal structures intertwined with a small amount of large coils.

The conformation of the arabinogalactan fraction of gum Arabic (AG) in solution has been investigated by Sanchez et al. using SANS, transmission electron and atomic force microscopies [37]. The AG fraction was recovered in a preparative hydrophobic interaction chromatography column, with a sequence of two NaCl solutions at 4mol.l^{-1}, 2 mol.l^{-1}, then distilled water (as described above). The form factor of the AG fraction in 50 mM NaCl D_2O solutions is reported in Figure I-12. At this salt concentration, the polyelectrolyte charges are screened and the correlation peak is suppressed.

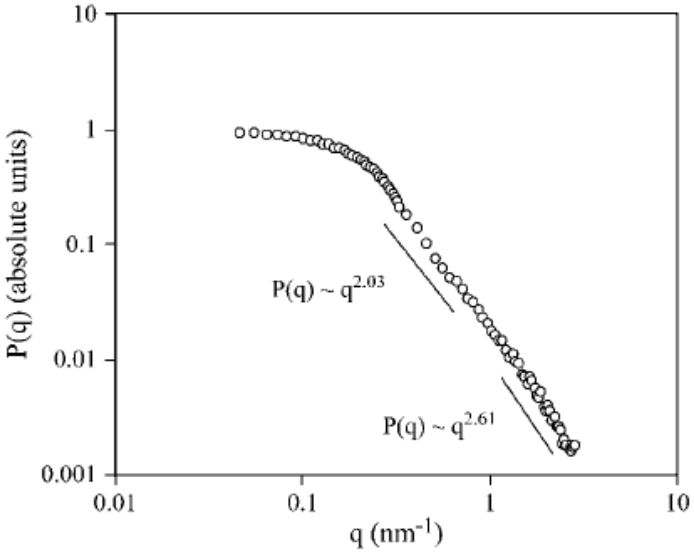

Figure I-12: Fraction 1 (AG) scattering form factor (1w/w%) at 25°C in D2O containing 50mM NaCl (from Sanchez et al., 2008) [37]

From the Guinier region (plateau in the low-*q* range) a radius of gyration of 6.4 nm was calculated. The pair distribution function of the gum fraction well compared with that of thin circular disks with a thickness of about 1.5 nm and a diameter around 20 nm. High-resolution microscopic measurements confirmed this structure. In the intermediate-*q* range, the scattering function decayed as q^{-2} interpreted by the authors as the signature of a disk-like particles population. Finally, in the high-*q* range the scattering intensity decayed as $q^{-2.61}$, which was attributed to a dense and ramified internal structure.

SANS was also used to study the structure of the arabinogalactan-protein conjugate fraction (AGP) of the gum by Renard et al. [38]. The fraction was also recovered using hydrophobic interaction chromatography (HIC). Samples for scattering experiments were prepared in the same way as in the study of Sanchez et al. (2008). HPSEC-MALLS coupled on-line with viscometer showed the presence of two types of conformations with globular and elongated shape depending on the size of the carbohydrate branches.

Small angle scattering experiments revealed the presence of an elongated conformation corresponding to a triaxial ellipsoid shape (Figure I-13).

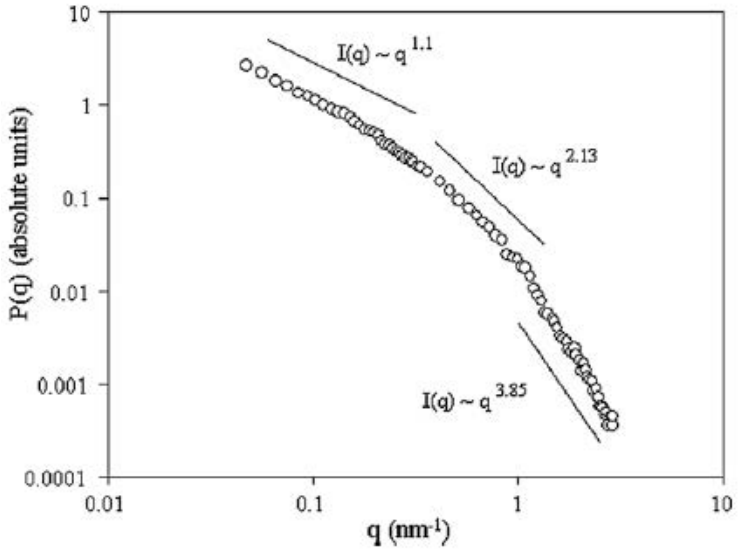

Figure I-13: Fraction 2 (AGP) scattering form factor (1w/w%) at 25°C in D2O containing 50mM NaCl (from Renard et al., 2012) [38]

In the low-*q* range the scattered function displayed a -1.1 power law, characteristic of a linear conformation. The -2.13 exponent in the intermediate-*q* range was attributed to a two-dimensional structure (disk or fractal). In the high-*q* the -3.85 slope which was identified as characteristic of a fractal structure of dimension 2.15.

The pair distance distribution function of this fraction revealed the presence of two maxima corresponding to half-distances of 10nm and 32nm, suggesting a two-dimensional conformation or the presence of a bi-dispersed population of particles.
Although TEM measurements of AGP moieties are quite sensitive due to their surface properties, Renard et al. have managed to perform AGP imaging using this technique. They noted different conformations according to their size, smaller species being spherical and larger ones more elongated [39].

The last HIC eluted fraction, the glycoproteins, was investigated using SAXS analysis by the same research team [40]. Samples for scattering experiments were prepared in an acetate buffer at pH=5 and 100mM of NaCl. SAXS measurements together with circular dichroism and TEM analyses led the authors to describe the shape of the glycoproteins fraction as an assembly of ring-like modules

Small angle scattering spectra of solutions of gum Arabic and its HIC recovered fractions displayed a correlation peak that was dependent on the gum concentration and disappeared when adding salt. This observation shows that electrostatic repulsions drive the structuration of gum moieties in solution. Moreover, studies focused on the characterization of gum fraction displayed structural differences between each fraction. The high molecular mass conjugate fraction (described as being responsible for the emulsifying and stabilizing properties of the gum) exhibits spheroidal and elongated shapes.

I-2-2 Gum arabic properties in solution

One practical advantage of gum Arabic is its high solubility in water, usually up to 50 w/w%. [31].

I-2-2-1 Rheology of gum Arabic solutions

Although gum Arabic is composed of high molecular weight molecules, the thickening property of gum Arabic remains limited compared to other common hydrocolloids. Indeed a 50 w/w% gum Arabic solution exhibits an apparent viscosity comparable to that of a 1.5 w/w% Xanthane gum solution [7]. Gum arabic solutions behaves as a shear-thinning fluid with a Newtonian plateau at large shear rates (>100s-1). In this regime, the apparent viscosity is an exponential growing function of the gum Arabic concentration, with a growth rate which is strongly dependent on the nature of the gum [13], [41]. Moreover an important increase in temperature will lead to an irreversible decrease of a gum solution viscosity due to denaturations of the protein-rich moieties [42].

More than shear-thinning gum arabic solutions are thixotropic, their response to deformation is time dependent. Sanchez et al. have observed an increase of the apparent viscosity as a function of

shearing time, especially significant as the shear rates is low [43]. The resting time of the solution prior to stress-strain measurements was also shown to increase apparent viscosity. This behavior was proved by the authors to result from a contribution of the solution sample surface rheology to the bulk stress response. This contribution arises from the adsorption process of proteinaceous species of the gum, building up with time a viscoelastic network at the sample-air interface. Replacing the water-air surface by an oil-water interface or working with a larger surface-to-volume ratio increases the time dependence of the rheological behavior, hence validating the proposed mechanism. In other rheological measurements performed by Li et al. (2009 and 2011), a PDMS film was deposited on the sample surface, to avoid evaporation [44], [45]. Although not mentioned by the authors, it is likely that this PDMS film also affected the adsorption rate of the proteinaceous species. In the first paper, the authors postulate the existence of an association-dissociation equilibrium of AG molecules which depends on the imposed shear rate of the resting time of the solution. The longer the resting time, the more compact are the AG aggregates, the faster is the deformation rate, the looser are the aggregates, tending towards a Newtonian behavior at large shear rates. This structuration mechanism is schematized in Figure I-14. Therefore, the history of the gum solution has a direct impact on its mesoscopic structure and its rheological behavior.

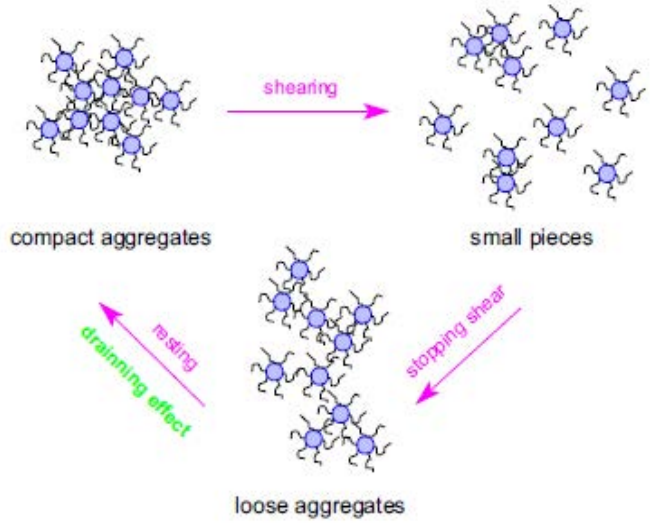

Figure I-14: State of gum arabic molecules with and without shear (from Li et al., 2009) [44]

In the second paper, the thixotropic property of the gum solution is investigated, submitting a 6 w/w% gum solution sample to a series of shear jumps inducing a sequence of stress jump responses [45]. The existence of an elastic contribution in the limit of low shear rate (<10s-1) is demonstrated due to the self-association mechanism of hydrocolloids, as well as a viscous contribution which takes over at larger shear rates, once the aggregates are dissociated. The stress relaxation curve after a sudden decrease of the shear rate clearly suggests a rapid development of an elastic network

resulting from molecular association within the gum. In fact, same experiments have been carried out removing the AGP moieties from the gum with single-walled nanotubes of carbon [46]. Without AGPs, the gum solution became fully Newtonian leading to the conclusion that this molecular association mechanism is driven by AGP and not to AG moieties.

I-2-2-2 Electrophoretic mobility

For the gum arabic in solution a value of approximately -1.5 µmcm/VS for the electrophoretic mobility was found above pH 4.5 and the value decreased close to zero as the pH was decreased to 2 (Figure I-15). This electrophoretic behavior was assigned to the carboxylic groups present on the polysaccharides glucuronic acid moities [47]. Moreover the pKa of glucuronic acid moieties is of 3.2 which is accordance with the measured electrophoretic mobility of gum Arabic as a function of pH (approximately 50% of the maximum electrophoretic mobility at pH 3) [48].

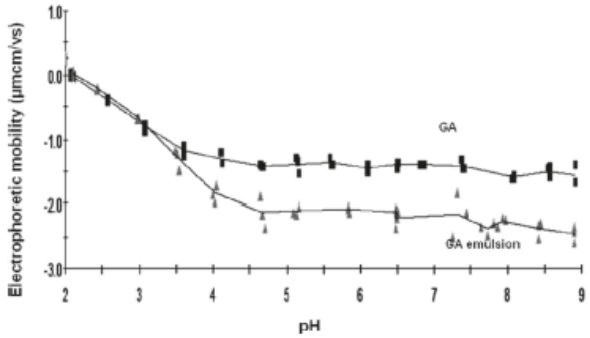

Figure I-15: Electrophoretic mobility of gum arabic and gum arabic strabilized emulsions as a function of pH (from Padala et al., 2009) [47]

I-2-3 Interfacial properties of gum Arabic

I-2-3-1 Emulsification and emulsion stability

It is now well admitted that the proteinaceous materials of the gum are responsible for its emulsifying properties. The treatment of the gum with a protease indeed inhibits its emulsifying activity [18].

Comparison of size exclusion chromatogram for gum Arabic solutions before and after emulsification has shown that a significant proportion of the high molecular mass species adsorbed at the interface as illustrated in Figure I-16 [18], [49]. It was also suggested that the proteinaceous moieties adsorbed at the interface and that carbohydrate groups extended into water to provide steric repulsion [24].

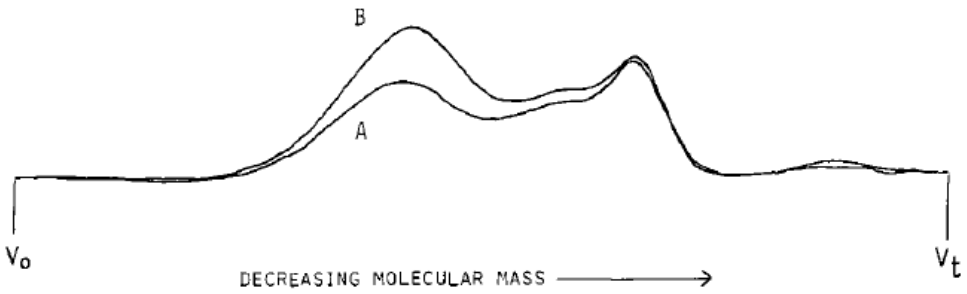

Figure I-16: Size exclusion chromatographic profiles of gum arabic in solution before (B) and after (A) emulsification as monitored by UV absorbance at 218nm (from Randall et al., 1988) [18]

Chikamai et al. demonstrated that heating a solution of gum Arabic at 100°C for 6h importantly destroyed the emulsifying properties of the gum whereas a temperature of 65°C did not really impact this property. [42]

Preparative size exclusion or hydrophobic interaction chromatography were used by Ray and coworkers to recover fractions of gum Arabic with different molecular masses or hydrophobicities [26]. It was found that the higher molecular mass fraction possessed the higher protein rate. The SEC recovered fractions were then used to stabilize oil-in-water emulsions at constant nitrogen concentration. Emulsions stabilized with fractions possessing the higher molecular weight and higher protein rate appeared to be the more stable. Also, it was observed that emulsion stability was increased when using a mixture of low and high molecular weight fractions. Finally, authors observed no differences in the zeta potential of the different emulsions and concluded that differences in stability were not originating from electrostatic effects.

Emulsifying properties of acacia gum were investigated by Buffo et al.. Using viscosity measurements on the oil-in-water emulsions they developed a model to estimate the thickness of the adsorbed species layer at the interface [50]. Layer thicknesses ranging from 190nm to 430nm were estimated according to the different gums studied (Senegal and Seyal). Gum processing such as pasteurization and demineralization were shown to enhance emulsion stability, due to an increase of molecular mobility and unfolding of the protein moieties at the interface.

Another emulsification stability study was carried out by H. Aoki and coworkers in 2007 [51]. Stabilities of oil in water emulsions stabilized by acacia senegal gum and mature gum Arabic were compared. According to accelerated stress conditions it was observed that emulsions stabilized with less than 20% of gum Arabic were not stable whereas when using a matured gum the stability was improved even with a concentration of 5%. Moreover when using a same concentration of native

gum or matured gum to stabilized an oil in water emulsion the authors observed that a matured gum provided emulsions with a finer droplet size. The same trend was observed in 2010 by O. Castellani et al. [52].

It was shown by Galazka and co-workers that protein unfolding is promoted under pressure [53]. Gum Arabic conjugates possess a small hydrodynamic radius with respect to their molecular mass suggesting a random coil structure unlike pectin for instance that is a semi-flexible hydrocolloid. Thus a high pressure could help the conjugates moieties to unfold and cover a greater surface at oil droplet interfaces (Figure I-17) [51].

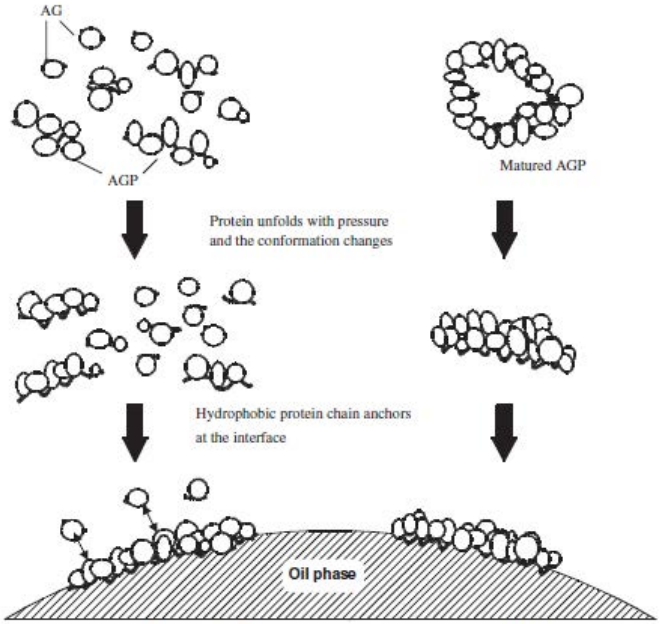

Figure I-17: Proposed model by Aoki et al. of gum Arabic adsorption onto the oil droplet (from Aoki et al., 2007b) [51]

Depletion flocculation in gum Arabic stabilized oil-in-water emulsion may occur. Indeed, the non adsorbed species present in the continuous phase of the emulsion can act as depletants. Flocculation is harmful for emulsions stabilities, it causes an increase in the creaming rate due to the increase in particles sizes and it may enhance coalescence events because droplets are brought into contact. Above a critical concentration non adsorbed polymeric molecules can increase the attraction between emulsion droplets because of an exclusion effect of those polymers from the region surrounding the droplets due to an osmotic effect [54]. In 2001 R. Chanamoi et al. studied the depletion flocculation of diluted emulsions by gum Arabic [55]. They varied the concentration of gum Arabic added to a tween 20 stabilized emulsion in order to identify the critical flocculation that induce droplets flocculation for a given emulsion droplets size distribution. The authors identified

that reducing the droplets size distribution increased the critical flocculation concentration. However no study was performed on the critical flocculation concentration for gum Arabic stabilized emulsion.

I-2-3-2 Adsorption isotherms and recovery of amphiphilic species

Randall et al. have determined the amount of gum adsorbed at oil-water interfaces by recovering the aqueous phase of a centrifuged orange oil emulsion, and taking the difference between the weight of gum in solution before and after emulsification [18]. A surface coverage of about 4 mg/m^2 was found for an emulsion stabilized with a 15 w/w% gum Arabic solution.

Yadav et al. have proposed a method for the purification of gum Arabic fractions adsorbing at oil droplets surface [12]. They used a tissue grinder to form gum Arabic stabilized hexadecane-in-water emulsions, let them cream, then broke these emulsions with an isopropanol solution to form a precipitate. After rinsing steps in various solvents, the precipitate was dried, dissolved in water, dialyzed against water and freeze-dried. In this study the emulsifying activity of different gums or gum sub-fractions was assessed from turbidity measurements. A correlation between the emulsifying activity of a gum and its lipid content was shown, although the amount of protein within each gum sample was not accounted for by the authors. The composition of the recovered gum sub-fractions displayed an important increase of the nitrogen content compared to that of the native gum, supporting the key role of proteins in the adsorption process. However, the emulsifying activity of the gum fraction recovered from the interface was not as important as expected. This result could be a consequence of a denaturation of the gum proteinaceous macromolecules during the separation process. The hydrophobic interaction chromatography analysis of this fraction indicated an increase in hydrophobicity compared to that of the native gum.

Another method to recover adsorbed species from gum Arabic stabilized emulsions was developed by M. Nakauma et al., using SDS surfactant to displace the proteinaceous material from oil-water interface [56]. Emulsions of medium chain triglycerides in gum Arabic solution were first centrifuged and the remaining emulsion was further mixed with a solution containing SDS and NaCl. The aqueous phase containing the displaced material was recovered and analyzed.

The surface concentration Γ (in mg/m^2) was deduced from the mass of adsorbed species and from the measurement of interfacial area (using a Static Light Scattering granulometer). Adsorption was found to be around 6mg/m^2 and nearly independent of GA concentration (Figure I-18A). In return, Γ increased significantly when NaCl was added to the aqueous phase before emulsification (Figure

I-18B), although some inconsistency can be pointed out in these results (the Γ value at the origin in Figure I-18B does not fit the value at a GA concentration of 10w/w% in Figure I-18A). Emulsions formed in the presence of salt were found to be more stable than emulsions prepared without. The increase of Γ thus seems to impact the metastability of gum Arabic stabilized emulsion.

However, it is important to recall that SDS denaturates proteinaceous materials by altering their tertiary structure [57]. Therefore, the method proposed by Nakauma et al. is limited to the determination of the amount of adsorbed species but is not suited to study their structural composition.

Another method to recover species that adsorbed onto the surface of oil droplets as a function of hydrocolloids concentration was used in 2008 by M. Nakauma and coworkers [56]. Emulsions of medium chain triglyceride in water were prepared. For gum Arabic stabilized emulsion the concentration range was from 1% to 10%. In order to recover the species adsorbed the authors mixed the emulsions with a solution containing sugar and NaCl. The mixture was then centrifuged for to recover the lower aqueous phase and this step was repeated three times. The remaining emulsion was further mixed with a solution containing SDS and NaCl. The SDS surfactant was used to displace the proteinaceous material from the interface. The lower aqueous phase containing the displace material was recovered. Recovered fractions containing the species that adsorbed at the interface were then analysed. Again the use of SDS precludes any relevant structural analysis due to denaturation [56].

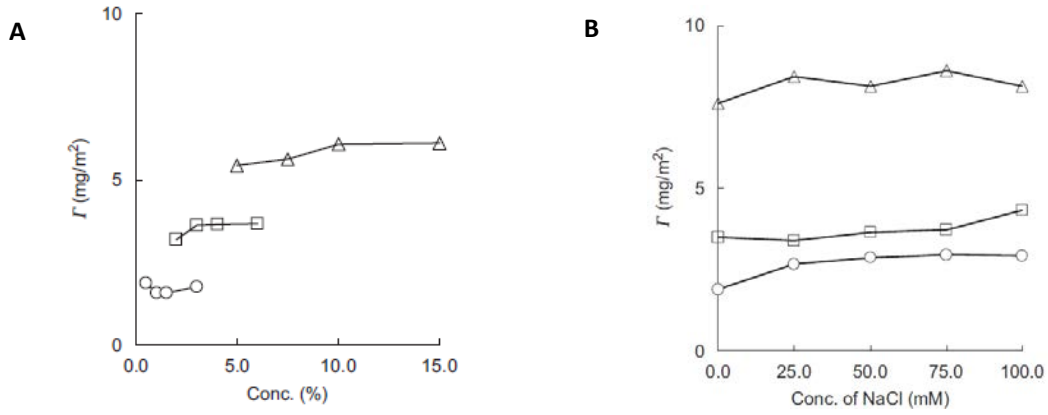

Figure I-18: Evolution of surface concentration in gum Arabic stabilized emulsions (triangle symbols) as a function of (A) GA concentration in solution, (B) Salt concentration for a concentration for a GA concentration of 10 w/w %). The pH of the aqueous phase was fixed at 3 prior to the addition to the oil (from Nakauma et al., 2008) [56]

Padala et al. (2009) have determined the mass of gum adsorbed at oil/water interfaces using size exclusion chromatography with refractive index detection. Chromatogram areas were compared between gum Arabic solutions prior to and after emulsification (Figure I-19A) and adsorption isotherms could be determined at different gum Arabic concentrations and two pH (Figure I-19B).

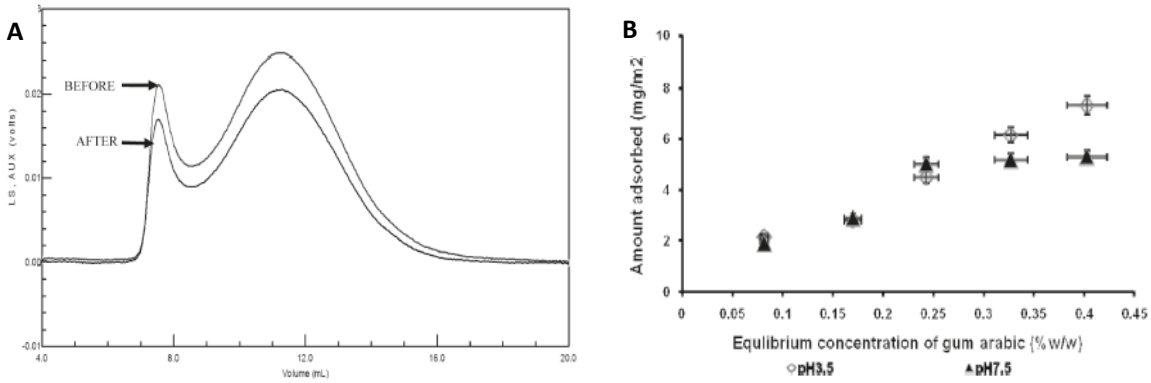

Figure I-19:(A) SEC elution curves of gum arabic at pH 7.5 monitored by RI and the supernatant recovered after preparing an emulsion. (B) Isotherms for gum Arabic adsorbing onto limonene oil droplets from solution at pH 3.5 and 7.5 (from Padala et al., 2009) [47]

One important finding from their work was that adsorbed species at the interface were not limited to the high molecular mass species as it had been described before [18], [49] but instead covered a wide range of molecular weight. Results showed a dependence of the mass adsorbed at the interface upon aqueous phase pH before emulsification. This depedence was ascribed to variations of the degree of protonation of uronic acid carboxylic groups. Indeed as seen before the isoelectric point of the gum is around pH 2. Hence, decreasing the pH increases the protonation of carboxylic groups, resulting in a less charged polymer. This results in a diminution of electrostatic repulsion at the interface enabling more species to adsorb. Considering the amount of adsorbed species at the interface Padala et al. argued that this high value (compared to other systems) was resulting from a conformational change of the macromolecules at the interface or from the formation of multilayers. Such a mechanism is supported by the tendency of gum arabic species to self-aggregate in solution [43], [44].

I-2-3-3 Rheology of gum Arabic stabilized interfaces

First thermodynamic quantity impacted by adsorption process is interfacial tension. According to Gibbs' relation, excess surface pressure is a growing function of surface concentration. Investigation of a possible correlation between interfacial tension and the nitrogen content (or protein content) of different acacia gums was carried out by Dickinson et al. [58]. Their results showed that two gums

with contrasted protein contents (1.86% and 7.5%) exhibited similar interfacial tension values after 15 hours of measurements. Moreover adsorption kinetics at short time did not indicate a correlation between interfacial tension and the protein content of each gum sample. Moreover no direct links were observed between the emulsifying capacity of a gum sample and its nitrogen content. Emulsion samples that displayed a lower size distribution and a better stability were the one with the higher and the lower nitrogen content. These observations are in contradictions with the study of Ray et al. Indeed here it is stated that there is not direct correleation between the protein rate and the emulsifying properties while in their study Ray et al. observed that they obtain the best emulsion metastability with the fractions having the higher protein rate [26].

Authors argued that regarding the effect of nitrogen rate on interfacial tension, two factors must be considered: the molecular weight of the molecules diffusing to the interface (the low molecular weight molecules diffusing more rapidly than the high molecular weight species) and the accessibility of the polypeptide moieties within the gum. Emulsion stabilized with a gum having the lower nitrogen content was quite fine and stable. The lowering of the interfacial tension with this sample was relatively quick due to the presence of low molecular weight molecules rapidly diffusing and adsorbing to the interface.

Interfacial tension seems to be a useful tool to probe the efficiency of a gum sample to diffuse towards an interface and to lower its energetic cost. Yet this measurement needs to be coupled with other analysis such as emulsifying activity (emulsion metastability) and chromatographic separation in order to fully probe the interfacial properties of a given gum Arabic.

Interfacial rheology measurements on interfaces formed with gum Arabic and other hydrocolloids were performed using the pendant drop method [20]. It was shown that that matured gum Arabic (heated gum Arabic with a higher amount of high molecular weight conjugate) had a higher surface activity compared to native gum as illustrated Figure I-20A. The lowering of the interfacial tension was slow compared to other system, indicating that gum Arabic needs a certain time to diffuse towards the interface, adsorb and rearrange itself [59]. The elastic behavior of the adsorbed film was also increased in the case of matured gum.

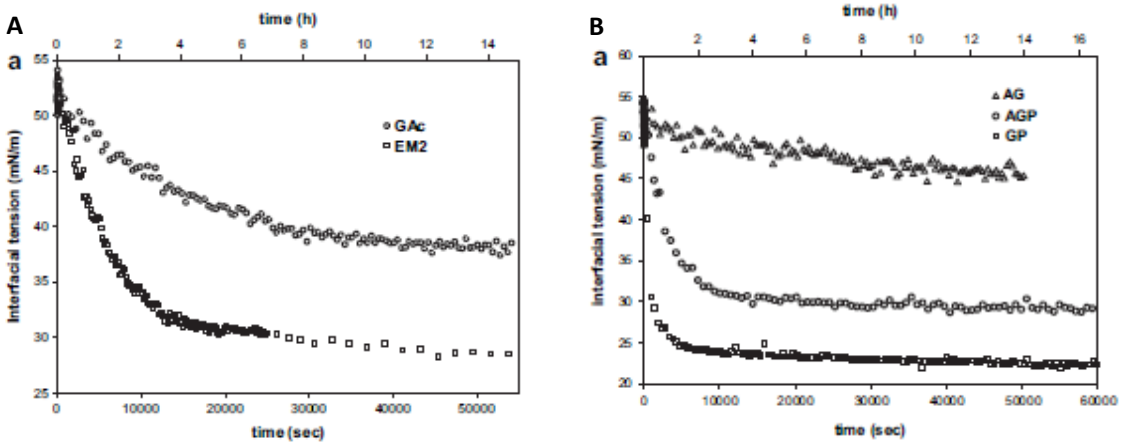

Figure I-20: Linear plot of interfacial tension kinetics of (A)control acacia gum (GAc) and matured acacia gum (EM2) (B)matured gum components (GA, AGP et GP) at the n-hexadecane-water interface (pH 4.5 and 20°C, 0.5/L) (from Castellani et al., 2010a) [20]

A pH sensitivity of the interfacial tension between n-hexadecane and an aqueous solution of gum Arabic was also observed. Interfacial tensions decreased when reducing the gum solution pH, while the dilatational modulus was increased. It was argued that neutralization of carboxylic moieties from the gum at lower pH favors an increase of the amount of adsorbed gum and thus a larger elasticity.

In the same study the authors fractionated a matured gum using hydrophobic interaction chromatography. They recovered three fractions corresponding to the gum conjugates, glycoproteins and polysaccharides. Pendant drop tensiometry analyses were performed with these fractions (Figure I-20B). As expected regarding the protein content of each fraction, glycoproteins were the most efficient to lower the interfacial tension, polysaccharides providing almost no interfacial activity.

Interfacial rheology addresses shear and dilatational viscosity measurements, which respectively consist in a shearing deformation of surface element at constant area and in a surface expansion or compression at constant shape.

Adsorbed gum Arabic films exhibit high interfacial shear viscosity as shown by Dickinson et al. [60]. A method to exchange the sub-phase liquid during interfacial shear measurement was developed by Dickinson et al. It was used to see whether the non-adsorbed species from the gum present in the aqueous phase of an emulsion provided a positive or negative impact on its stability. It was observed that if the aqueous phase was diluted almost no change in surface viscosity occurred on a long time scale. These results indicated that some species from gum Arabic strongly adsorbed at an oil/water interface.

Interfacial rheological properties of adsorbed gum Arabic and modified starch at water/carvene interfaces were studied by Erni and co-workers [61]. It was demonstrated that acacia gum exhibits a significant shear elasticity of the interface.

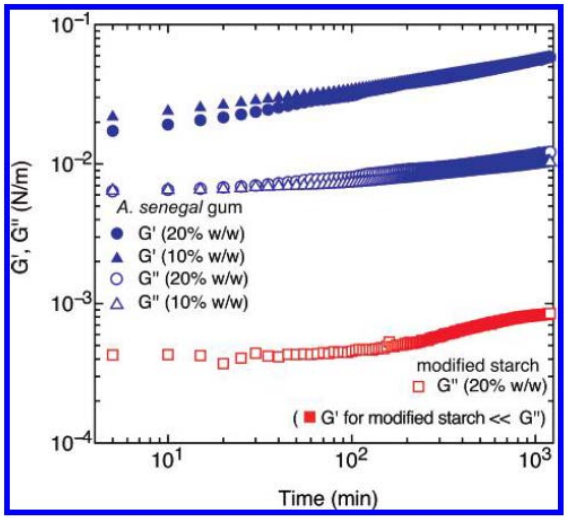

Figure I-21: Time dependence of the interfacial shear moduli G' (storage) and G'' (loss) of acacia senegal gum and hydrophobically modified starch at the oil/water interface (T=20°C, deformation amplitude γ_0=1w%). The storage modulus G' for modified starch was below the detection limit (from Erni et al., 2007) [61]

The authors observed that acacia gum layers were elastic over the whole range of time studied with the storage modulus above the loss modulus (Figure I-21). Moreover, measurements of the shear moduli frequency dependence revealed that gum Arabic layers remained elastic at all frequencies measured with a weak dependence on the oscillation frequency. Authors suggested an analogy with either “soft glassy” materials or gel networks.

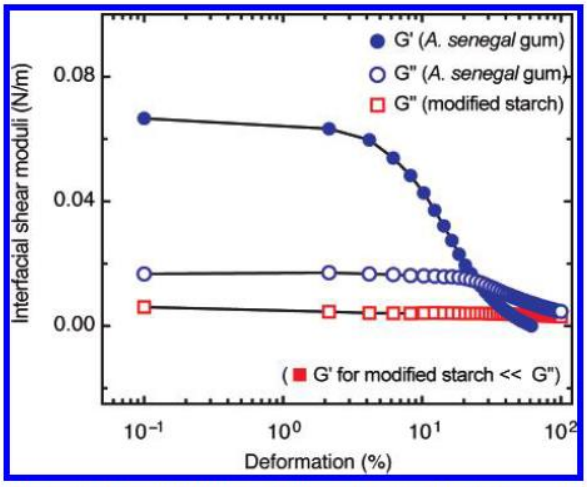

Figure I-22: Deformation dependence of the interfacial shear moduli G' and G44 of acacia Senegal gum and hydrophobic ally modified starch at the oil/water interface (T=20°C, ω=1 rad.s-1, interface age t=20h) (from Erni et al., 2007) [61]

As seen in Figure I-22 deformation dependence of interfacial moduli for gum Arabic layers showed a strong decrease of the elastic modulus above 1-2% deformations. This behavior suggests that the adsorbed film is a fragile network with a structural breakdown above a certain deformation rate. The authors thus concluded that the mechanical stability of the interfacial film must play a significant role in the stabilizing properties of the gum [61].

Spreading capacity (capacity to increase surface pressure when decreasing interfacial area) at air/water interface of different hydrocolloids using langmuir compression isotherms was studied by Castellani et al [62]. Matured gum had better spreading capacity and an acidification of the aqueous phase improved the spreading of native gum Arabic. More over the authors used AFM to measure the thickness of adsorbed film layers at air/water interface. According to their findings a decrease in the pH increased the thickness of the film layers (150nm at pH 3.1 over 30nm at pH 4.5). The AFM imagining of the adsorbed films at pH 3.1 revealed the presence of aggregates with localized zones of higher thickness. To complete their study, the authors performed ellipsometry measurements on adsorbed gum Arabic films at the air/water interface. Differences in ellipsometry angle for adsorbed film at pH 4.5 and pH 3.5 correlated with the thickness measured with AFM.

Interfacial tension and rheology studies that were carried out on gum Arabic samples revealed a non straightforward relationship between protein content and adsorption behavior, which contradicts other results showing the gum fraction having the higher protein rate providing the best emulsion metastabilities. The adsorption behavior of gum Arabic at oil/water interfaces appeared slow compared to other system and revealed a characteristic behavior of this complex system where molecules need time to adsorb and rearrange at the interface. Moreover, gum Arabic adsorbed films present an elastic behavior that might originate from the formation of a fragile network at the interface. Finally, these studies showed a dependence between gum adsorption and a decrease in pH of gum solutions, with increased interfacial properties (lower interfacial tension and higher elasticity).

I-2-4 Conclusion and thesis objectives

Arabic gum has been extensively studied over the past 40 years. This literature review shows the extremely heterogeneous structure and composition of the gum, as a complex mixture of polysaccharides and protein moieties. Two types of chromatographic separations (size exclusion and

hydrophobic interaction) coupled with different types of detection were used on different gum Arabic samples in order to improve the understanding of the molecular composition of the gum. A description of the gum containing three fractions was proposed, consisting of a bulk fraction composed of polysaccharides moieties, a fraction of protein-polysaccharides conjugates and a smaller fraction of glycoproteins [7], [15], [21], [24], [25], [27], [63]. For each fraction (separated through preparative hydrophobic interaction chromatography), their hydrodynamic radius, molecular masses, polydispersity, viscosity, emulsification behavior, interfacial tension, structure in solution were thoroughly investigated.

The role of the proteinaceous moieties of the gum on its interfacial properties was evidenced and it was shown that the high molecular masses species appeared to preferentially adsorb at the interface proving steric repulsion [18], [24], [64]. Although gum Arabic is composed of polyelectrolytes, ionic repulsions were not described as the main mechanism for emulsion droplets stabilization [26].

Gum Arabic amphiphilic species were shown to strongly adsorb at the interface and a dilution of a gum Arabic stabilized emulsion does not seem to affect its metastability [60]. One direct consequence is that the non-adsorbed species of gum Arabic do not influence the emulsion stabilization mechanisms. Interfacial rheology measurements with gum Arabic or its fractions displayed an elastic behavior of the adsorbed film when submitted to perturbations [60], [61]. This macroscopic observation reflects a given structuration of the interface which needs deeper insights.

The effect of physico-chemical parameters such as pH or salinity of gum Arabic solutions on the metastability of the emulsion, or the adsorption behavior have been demonstrated (interfacial tension measurements, isotherms of adsorption) [47], [56], [62], [65].

Despite this impressive amount of work, many questions remain largely open as recently reviewed by Sanchez and coworkers [13]. This notably includes two points treated in this thesis:

- A unified description of structures displayed by the various species composing gum Arabic, both in solution and at interfaces
- A comprehensive understanding of the impact of formulation on these structures and thus on emulsifying properties

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Chapter II- Gum Arabic: composition and multi-scale structure in solution

Abstract.

Gum Arabic is a natural exudate from acacia trees, widely used to stabilized dispersed systems such as oil-in-water emulsions. The gum is commonly described in the literature as a mixture of polysaccharides, protein-polysaccharides conjugates and glycoproteins. Here, we investigated the structural composition of gum Arabic in solution using a two dimensional chromatographic separation, consisting of size exclusion followed by hydrophobic interaction. We show that neither size nor hydrophobicity are truly efficient separation criteria while the combination of both unveils the different species composing gum Arabic. We notably identified two Arabinogalactan-peptide populations, one of them smaller with a higher protein content and hydrophobicity. Also four glycoprotein populations were identified of different sizes and content in hydrophobic amino-acids. These two types of populations are the building blocks composing larger Arabinogalactan-protein conjugates. Using both neutron and X-ray small-angle scattering on both gum Arabic at various concentrations and ionic strength and size-fractions, we propose a concentration-dependent multi-scale structure for gum Arabic in solution.

Introduction.

Gum Arabic, or acacia *Senegal* gum, is an exudate produced by acacia trees, which shields them against insects, molds and drought. Highly water soluble, gum Arabic has been under extensive use since the dawn of civilization with an extensive array of applications [1], [2], notably as a binder and emulsifier. This natural hydrocolloid is composed of a complex mixture of biopolymers. In order to understand gum Arabic interfacial properties, its composition and associated structures have been extensively studied. The gum was first identified as a heterogeneous mixture of protein and polysaccharides differing in their molecular weight and hydrophobicity [3]–[6]. Chromatographic analysis has been the most employed technique in the literature to characterize gum Arabic structural composition. Gum species were first separated through their molecular weight using size exclusion chromatography [6], [7]. It was shown that the gum presents a continuum of molecular weight ranging from 5.10^6g.mol^{-1} to 1.10^5g.mol^{-1} with a mean molecular weight around 5.10^5g.mol^{-1}. Gum Arabic polypeptides moieties were described as being associated with the higher molecular weight fraction. Hydrophobic interaction chromatography separations was later performed on gum Arabic, enabling the sorting of species according to their hydrophobicity. From these separations the gum was described as a mixture of three fractions: an arabinogalactan rich polysaccharide fraction constituting the bulk of the gum (AG) (88%), a polysaccharide-protein conjugates fraction (AGP) (10%) and a fraction of glycoproteins (GP) (2%) [8]–[14]. Yariv reagent was used on each of these recovered fractions and it was observed that

protein/polysaccharide conjugates were present in each [10]. The relative proportion of each species for a given gum Arabic sample depends on the geography, soil and age of the threes, however the overall chemical and physico-chemical characteristics are quite constant [11], [15]–[17]. A two dimensional separation of gum species was performed using size exclusion chromatography as the first dimension followed with a hydrophobic interaction separation. Renard et al. showed that the gum contain a hydrophilic fraction (associated with the lowest protein content) with a small polydispersity in size, whereas the other fractions (with higher protein content) displayed a broad range of sizes [13].

Gum Arabic conjugated moieties structure was extensively studied and several models were proposed. The first model proposed described the gum as wattle blossom shape with a polypeptide chain bearing approximately five branches carbohydrate moieties [7]. Then the gum as described as a twisted hairy rope by Qi et al while Goodrum and co-workers proposed a symmetric representation of the gum conjugates based on a palindromic polypeptide sequence [18], [19]. Finally, Mahendran and co-workers proposed a slightly different representation with the main difference arising from the molecular weight of the arabinogalactan units but in accordance with previous proposed model [20].

Small angle neutron and x-ray scattering measurements were performed on gum Arabic and its fractions. First, these measurements were performed on unfractionated gum Arabic by Dror et al., with variations of the gum concentration and salt concentration. A correlation peak on gum aqueous solutions without water, showed the presence of a correlation length between the scattered moieties due to ionic repulsions. Then Renard, Sanchez and coworkers recovered three fractions, through gum Arabic separation by hydrophobic interaction chromatography, which were analyzed by SAXS and SANS, leading the authors to propose models to describe each fraction. The conjugates fraction (AGP) was described as a thin ellipsoid and the AG fraction as a thin disk.

In this study we first performed a two dimensional separation of gum Arabic moieties to further characterize its composition in molecular weight and hydrophobicity. Size exclusion chromatography was used as the first dimension separation and five fractions with various molecular weight were collected, followed with a hydrophobic interaction separation. The second step consisted in the structural characterization of gum Arabic and its fractions using small angle neutron and x-ray scattering experiments. Both steps from this study led us to propose a multi-scale structure for gum Arabic in solution at different concentrations.

Results.

Chromatographic separation:

Chromatographic analysis of gum Arabic has been implemented in order to investigate its structural composition. In order to improve our knowledge of the molecular mass and hydrophobicity breakdown within the gum a two-dimensional chromatographic analysis has been further carried out.

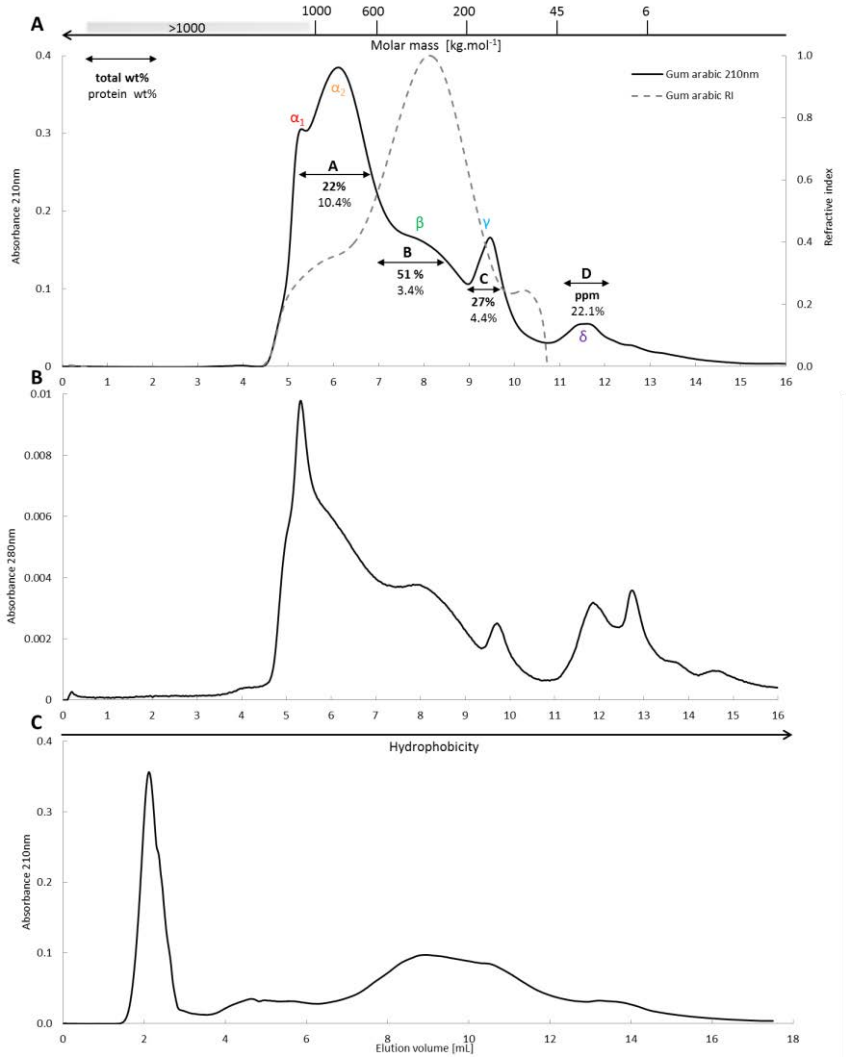

Figure II-1: (A) Size exclusion chromatogram at 210nm, (B) at 280nm and (C) Hydrophobic interaction chromatogram of a 3w/w% aqueous solution of gum Arabic. Dotted line: Refractive index detection. (A)Weight percentages of recovered fraction using a preparative size exclusion column (same column filling, larger dimensions) are indicated as well as the protein rate for these fractions. Scale for molecular weight as a function of elution volumes is displayed on the top of the chromatograms and was obtained using branched dextrans of known molecular weight (universal calibration curve). (B) Less hydrophobic species of the gum are not retained by the column and species eluted last are the more hydrophobic ones as indicated by the scale on top of the chromatogram. (SEC column: Biosuite 450A SEC Waters ®, HIC column: Biosuite Phenyl HIC Waters ®)

Figure II-1A displays the size exclusion chromatogram of our gum Arabic using both a UV detection at 210 nm, which is mostly sensitive to the peptide bond [21], [22], and a refractive index detection, which is proportional to concentration. This chromatogram is similar to previously published gum Arabic chromatograms [16], [13], [23], which ensures the representativity of our gum Arabic batch. We observed that gum Arabic displays a broad range of molecular weight ranging from 6 $kg.mol^{-1}$ to more than 1000 $kg.mol^{-1}$. From the refractive index signal, it can be stated that the gum is predominantly composed of the intermediate molecular masses, whilst the higher molecular masses (peak α_1 and α_2) make up about 15 wt% of the gum. These data are consistent with the weight fractions of the different gum Arabic fractions obtained through a similar, but preparative, chromatographic separation (A, B, C and D). The protein weight fractions were also determined and display variations ranging from 3.4 to 22.1% between the different fractions. UV detection at 280 nm is mostly sensitive to the cyclic amino acids (tryptophan and tyrosine). Thus the size exclusion chromatogram of gum Arabic at this wavelength gives information on the hydrophobic amino acid rich fractions. Fraction α_1 (high molecular weight) happen to be the more intense while fraction α_2 is way less intense as compared to the signal at 210nm. Another important observation is that peak γ (135$kg.mol^{-1}$) is less intense than peak δ, which is actually split into two peaks unlike the chromatogram obtained with a 210 nm detection. This indicates that polypeptides of fraction δ contain more hydrophobic amino acids.

The protein content measured in all fractions (from size exclusion separation) suggests that the various species of gum Arabic may display amphiphilicity. Hence, hydrophobic interaction chromatography was used to probe the hydrophobicity of gum Arabic, which results from its interaction with a phenyl grafted column. Unlike other studies [12], [13], [24], experiments have been performed with a continuous gradient of salt concentration and the resulting chromatogram is displayed in Figure II-1B. The first peak corresponds to the dead volume of the column and thus to species that are not retained in the column. This peak is narrower and more intense than the rest of the chromatogram, which suggests that there is a rather homogeneous population of hydrophilic species in gum Arabic. In return, the rest of the chromatogram displays a broad elution profile, which emphasizes the heterogeneity of the gum as a continuum of species of increasing hydrophobicity. The dual chromatographic characterization of gum Arabic highlights its complex composition in size and amphiphilicity and thus calls for further investigation of the interfacial composition of oil/water interfaces in the presence of gum Arabic.

Two dimensional separation:

Renard and co-workers performed a two dimensional separation of gum Arabic species using hydrophobic interaction chromatography followed by size exclusion chromatography [13]. From the

hydrophobic interaction separation the authors recovered three fractions of contrasted protein rate (fraction I: 1.1 w/w%, II: 9 w/w% and III: 24.6 w/w%). Then each fraction was injected in a size exclusion chromatographic column (0.04w/w%). Results are presented in Figure II-2. The SEC refractive index signal of the whole gum is similar to that we obtained (Figure II-1A), with the presence of two distinct populations: a population of high molecular weight molecules representing 10-15w/w% of the gum and another population representing the bulk of the gum with a smaller molecular weight. The less hydrophobic fraction (fraction I) was found to be rather homogeneous with an elution in the intermediate molecular masses. From the refractive index signal this fraction seems to represent the bulk of the gum. The other two fractions (fraction II and III), were found to be more heterogeneous in molecular weight especially in the case of the more hydrophobic fraction.

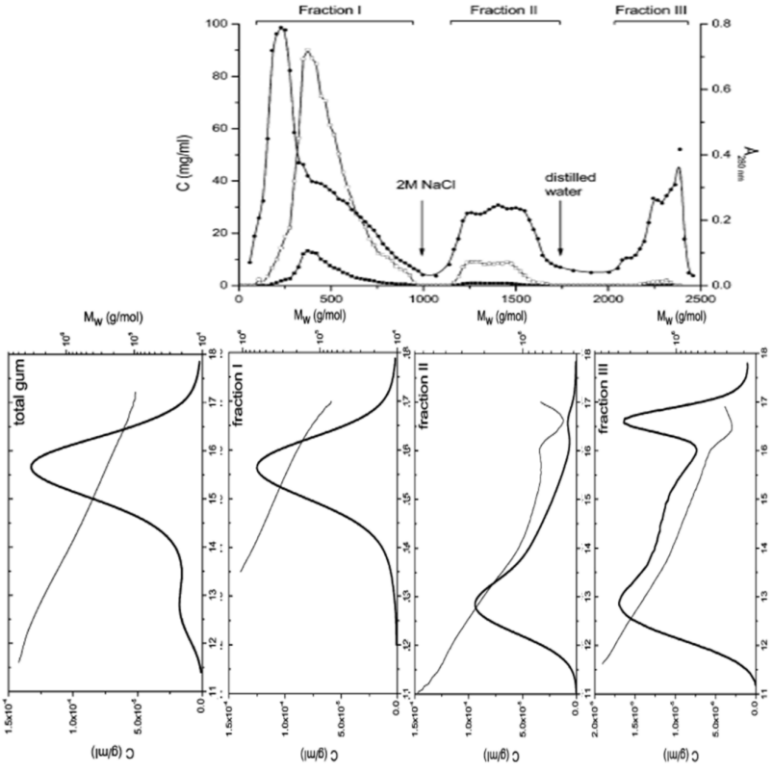

Figure II-2: Two-dimensional separation of gum arabic with hydrophobic affinity as the first dimension column and size exclusion as the second dimension. From [13].

This first study prompted us to further investigate gum Arabic composition with a two-dimensional separation, starting with size exclusion chromatography followed by hydrophobic interaction chromatography. Size exclusion chromatography on gum Arabic was better resolved. Five fractions were collected by a size exclusion separation differing in their molecular weight using a fraction collector as indicated by the Greek letters on Figure II-1 (α_1: 5.2mL, α_2: 6.04mL, β: 8mL, γ: 9.5mL, δ: 11.5mL). Once collected these fractions were concentrated prior to injection in the second dimension column by freeze-

drying. The dissolved fractions were injected in the size exclusion column (first dimension) for a control. The resulting chromatograms are presented in supporting information in Figure III-1. Each chromatogram presents a single peak centered around an elution volume. The five fractions differing in their molecular weight ($F\alpha_1$: >1000kg.mol^{-1}, $F\alpha_2$: 800kg.mol^{-1}, Fβ: 420kg.mol^{-1}, Fγ: 135kg.mol^{-1}, Fδ: 23kg.mol^{-1}) were then separated in the hydrophobic interaction column. The two-dimensional separation resulting from this procedure is reported in Figure II-3. A UV at 210nm detection was used for the two dimensional separation. Fractions γ and δ were more concentrated than the others (otherwise no signal could be detected).

The first important finding drawn from the two-dimensional separation of gum Arabic molecules is the heterogeneity of a class of molecular weight molecules in hydrophobicity. Indeed for each fraction no narrow peak is observed unlike what was observed by Renard et al for their less hydrophobic fraction (FI). The vertical line after 2mL of elution drawn on the hydrophobic interaction chromatograms of Figure II-3 denotes the theoretical dead volume of the column (limit between non retained (before the line) or retained (after the line) species in the column). It can be observed that only fractions α_1, α_2 and β contain some species with almost no hydrophobicity since those are not retained in the column. Whereas in the case of fractions γ and δ, no species were eluted before 2mL. Thus all species contained in these fractions (γ and δ) were retained in the column and exhibited hydrophobicity.

The higher molecular masses (fraction α_1) molecules from the gum are composed of the more hydrophobic species (elution between 11 and 17 mL) in addition with the hydrophilic fraction from the first peak (before 2 mL). The second fraction contains mainly moieties with intermediate hydrophobicity (elution between 6 and 14mL). The third fraction corresponding to the shoulder on the size exclusion chromatogram is composed of species with a broad range of hydrophobicity. We observed that for fraction α_2 and β the intensity of the non-retained species peak (hydrophilic species) is more intense then the rest of the chromatogram. This observation supports the results of D. Renard et al. Indeed fraction α_2 and especially fraction β represent the intermediate molecular weight molecules, which were found to be the less hydrophobic (fraction I). Fraction γ seems to contain species with relatively low hydrophobicity (peak at 5mL) and some species with the highest hydrophobicity (between 14 and 16mL). Finally the fifth fraction only shows two peak around 3mL as well as a broad range of hydrophobicity (low intensity nearly flat profile). UV detection being mostly sensitive to protein moieties it indicates in which fraction (sizes) the most hydrophobic amino acids of the gum are present. These ones appear to

be mostly present in the high molecular weight species (fraction 1 >1000kg.mol^{-1}) but also in each every fraction (UV absorbance in the high elution volumes).

This two-dimensional separation of the gum helped to improve the understanding of the hydrophobic interaction chromatogram of the gum. Additionally this study highlighted the heterogeneous nature of this complex mixture.

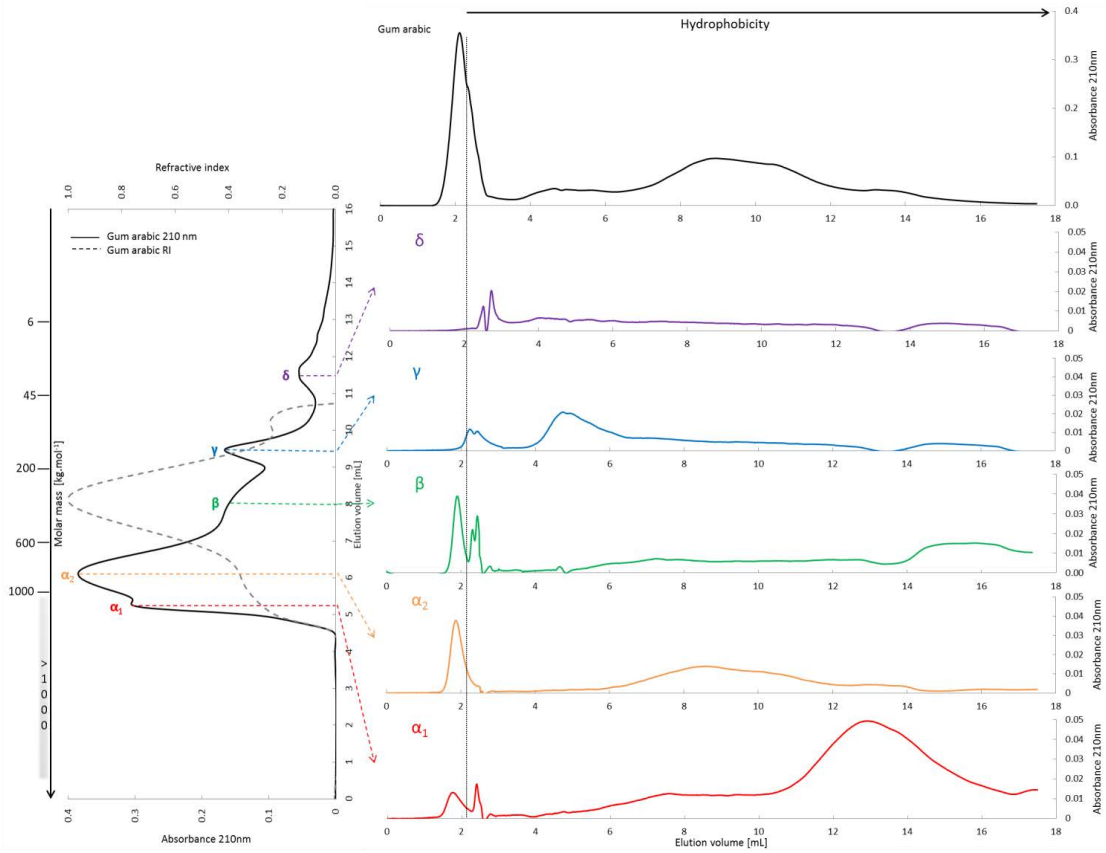

Figure II-3: Two-dimensional separation of gum arabic with size exclusion chromatography as the first dimension column and hydrophobic interaction as the second dimension. Five fractions were collected by size exclusion chromatography with a narrow volume collection (120µL). (α_1: >1000kg.mol-1, α_2: 800kg.mol-1, β: 420kg.mol-1, γ: 135kg.mol-1, δ: 23kg.mol-1).

Structure of gum Arabic probed by SANS and SAXS

Given the colloidal nature of gum Arabic species, scattering methods are well-suited due to their non intrusive characterization. Light scattering methods have been used and suggested an association behavior in solution of gum Arabic species [25]. However, the colloidal length scales are more adequately studied through small-angle scattering using X-ray (SAXS) or neutron beams (SANS). For isotropic samples, the structure is described in the reciprocal space through intensity versus q spectra, q being the magnitude of the scattering vector, expressed in inverse length units. The low-q range thus corresponds to large correlation length scales while the high-q range corresponds to small correlation length scales. An important difference between these two similar methods lies in the origin of contrast, which relates to electronic clouds for SAXS and to nucleus types for SANS. In particular, the use of deuterated molecules is a convenient way to tune the SANS contrast. Two groups have published important contributions on the structure of gum Arabic in solution. Dror, Cohen and coworkers [26] used both SANS and SAXS to study gum Arabic aqueous solutions at various ionic strength and concentrations. Renard, Sanchez and coworkers used either SANS or SAXS in a series of four papers dedicated to solutions of gum Arabic fractions obtained through hydrophobic interaction chromatography [27]–[30].

In the present work, gum Arabic structures in solution have been investigated within a more extended q-range, which allows the probing of larger structures, using a dual SAXS and SANS characterization on identical samples. Gum Arabic fractions obtained from size-exclusion chromatography have been also characterized with these two scattering techniques. First of all, two SANS spectra of both our gum Arabic batch (Caragum international ®)and another batch from the same source as that studied by Renard et al. (CNI company ®) were collected, at the same mass fraction 0.5% in D_2O and 0.02M NaCl of ionic strength. The two scattering curves are nearly identical (see Figure II-12 in SI), which suggests that from a neutron scattering viewpoint, the two gums are comparable. Strikingly, we observe large length scale correlations through an abrupt increase of the scattered intensity with decreasing q (Figure II-4). In this low-q range, the intensity varies like q^{-3}. This intensity upturn at low q was identically observed on the SANS spectra of gum Arabic at the same concentration but without salt (Figure II-4). The signature of large correlation lengths was not observed by Renard and coworkers since it occurs in a lower q-range than that they studied. In the work of Dror and coworkers, this low-q range was only investigated for a 5 w/w% gum Arabic solution and a strong intensity increase was also observed but with a q^{-2} power law. Importantly, filtrating our aqueous solution with a 200 nm filter did result in any visible changes on the SAXS spectra (see Figure III-13 in SI), which suggests that these large scale structures are highly flexible and possibly results from a self-assembly behavior as previously suggested in light scattering studies [31]. Another

feature observed on the SANS spectra is the development of correlation peak in the intermediate-*q* range (red and blue spectra), which is screened under salt addition (black curve) as already observed in previous works [26]. Finally, the small length scale behavior in the high *q*-range does not follow the Porod law in q^{-4} for smooth interfaces but rather a q^{-2} law, which can correspond to several structures such as Gaussian chains, a porous network with a fractal dimension of 2 or two-dimensional objects such as disks [32]. However, the signal-to-noise ratio is too high in this *q*-range to consider a finer analysis.

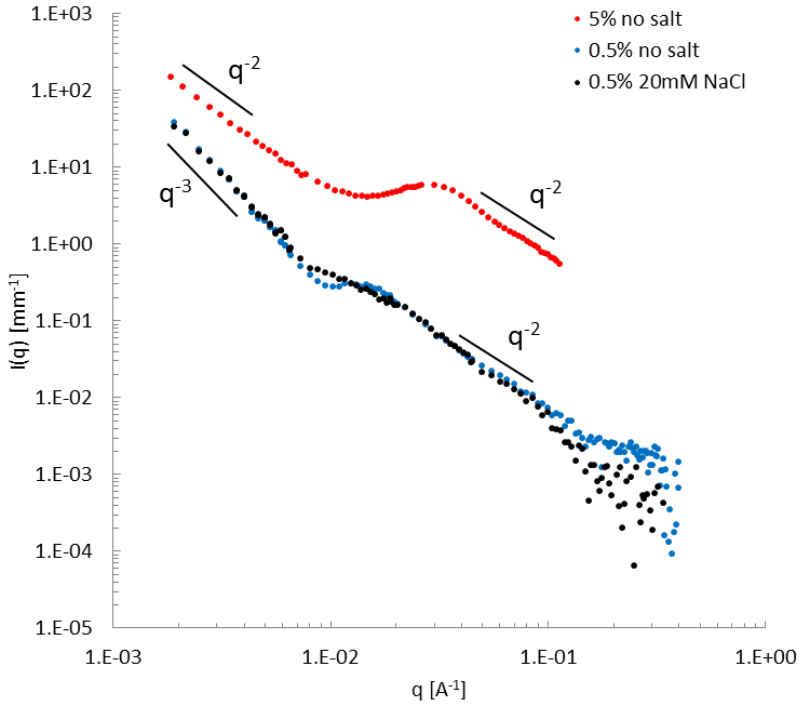

Figure II-4: Small angle neutron scattering spectrum of gum sample at 0.5w/w% or 5w/w% with (20mM NaCl) or without salt. Samples were measured in D_2O. Data from gum spectra at 5w/w% from Dror et al., 2006 [26].

We therefore also performed SAXS measurements at various concentrations and ionic strength over an extended *q*-range compared to the work of Dror et al. The results are displayed in Figure II-5. In the high-*q* range, all curves overlap when the intensity is normalized by the concentration, which indicates a self-similar structure at small length scales, independent of concentration or ionic strength. A $q^{-2.1}$ power law is followed by a $q^{-2.5}$ power law, providing a more resolved behavior in that range than the SANS spectrum. Such a signature is more compatible with form factors of mass fractals rather than two-dimensional objects. Furthermore, the presence of oscillations suggests that well-defined correlation lengths are present over these few nanometers length scales. In the intermediate-*q* range, a correlation peak is observed. Its magnitude decreases with the ionic strength, which is expected for colloidal objects interacting through long-range ionic repulsions. Consistently with previous reports, 20 mM and 50 mM NaCl ionic strengths were sufficient to suppress this correlation peak. In no added salt conditions (Figure

II-5B), the *q*-abscissa of this structure peak varied as the gum concentration to the power 1/3, as also observed by Dror et al [26]. This scaling law indicates repulsions between colloidal objects rather than within a concentrated polyelectrolyte matrix [26]. For the 0.5%, the correlation distance is 44 nm, while for the 5% solution it is 21 nm. Since the correlation peak significantly broadens at a 10 w/w% gum concentration, we can deduce that the onset of the overlap occurs at a gum concentration ranging between 5 and 10 w/w%, corresponding to 15 to 20 nm correlation distances.

At low gum concentrations and 20-50 mM NaCl, the correlation peak disappears and the intensity rather reaches a constant value in the limit of low *q* values. This indicates a finite size of the scattering objects, with a radius of gyration that could be estimated to 7nm, using the $Iq^2(q)$ plot in which $q_{peak}R_g=3^{1/2}$ (see Figure II-6). This value is compatible with the spacing deduced from the correlation peak *q*-abscissa at the overlap concentration, which means that these finite objects are the charged objects. In the corresponding SANS spectra, the intensity never reaches a constant value due to its strong upturn in the low-*q* range, which is not observed in the SAXS spectra. Only a slight increase of the scattered intensity at low *q* could be detected for larger gum concentrations. This SANS/SAXS mismatch is uncommon and calls for an explanation.

We verified that SAXS spectra of both H_2O gum solutions and D_2O gum solutions were identical. The SANS/SAXS mismatch is thus unrelated to any slight interaction differences in the two systems, which may manifest in the vicinity of a phase transition. This leaves only another explanation related to differences in contrast distribution within the scattering material. This corresponds to a heterogeneous scattering system, the parts of which display important contrast differences with the solvent, between SAXS and SANS experiments. SANS experiments were performed by dissolving gum Arabic in deuterated water (D_2O) so that the contrast difference mainly originates from the hydrogen/deuterium ratio, while in SAXS experiments the contrast difference originates from differences in electron density. SANS/SAXS mismatch likely results from two superimposing phenomena, which both highlight the polypeptide backbone compared to the polysaccharides. Firstly, hydrogen-deuterium exchange is expected on labile hydrogen [33], which are present on both hydroxyl (sugars) and amine groups (proteins). This will lead to a contrast decrease between polypeptide/polysaccharides and deuterated water, which will be more pronounced for the polysaccharides since they contain more hydroxyl moieties. Secondly, polysaccharides are likely more hydrated than at least some parts of the polypeptide chains, which possess hydrophobic amino-acids and secondary structures. This will drastically reduce the contrast of polysaccharides except at molecular length scales, which probe directly hydration. We can thus conclude

that the intensity upturn in the low-*q* range indicates large scale structures of inner parts of the gum structure, which are likely polypeptide chains.

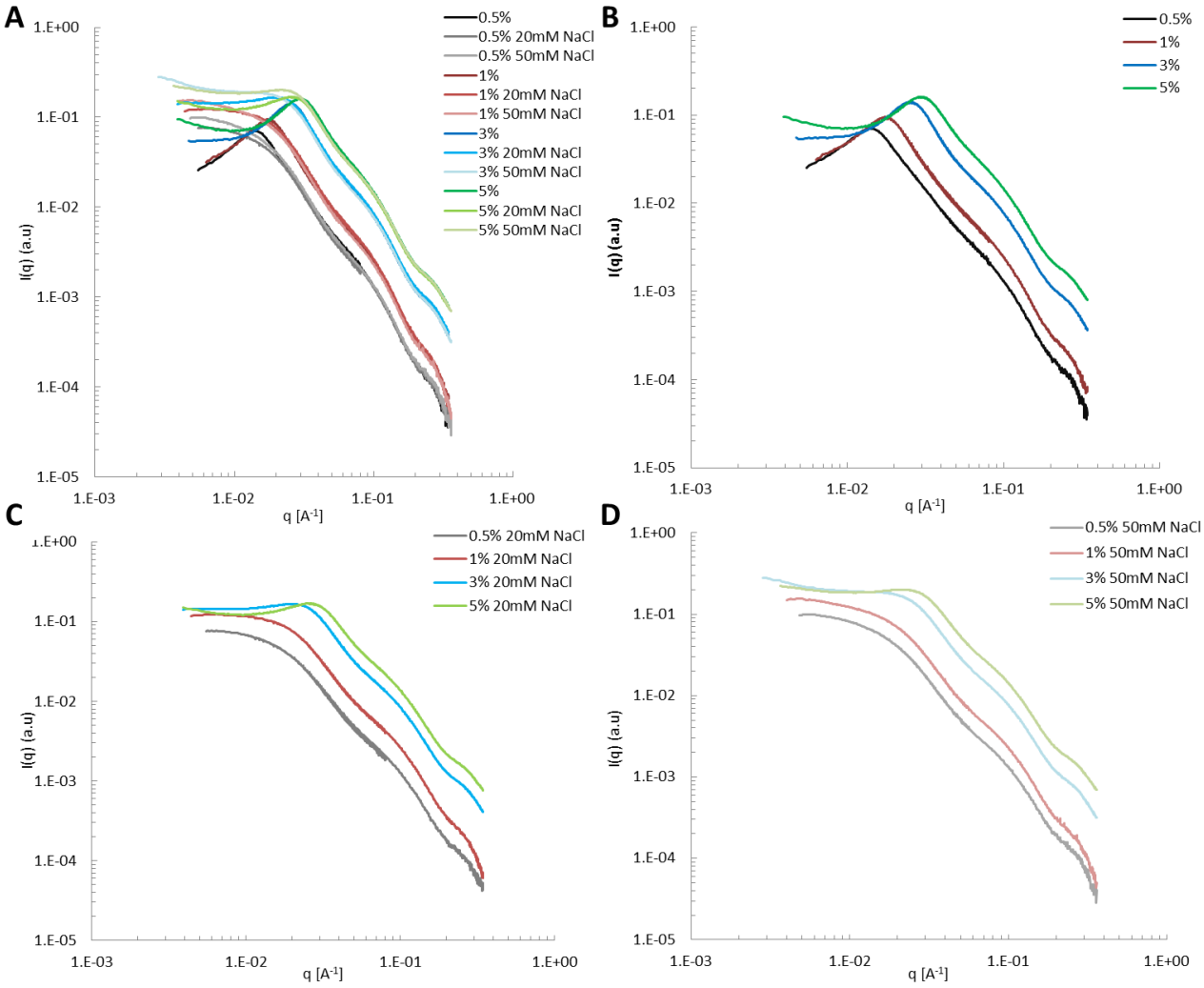

Figure II-5: Small angle x-ray scattering spectra of gum arabic at different gum and NaCl concentrations (A): superimposition of all conditions, (B): no salt, (C): 20mM NaCl and (D): 50mM NaCl. Samples were prepared in distilled water.

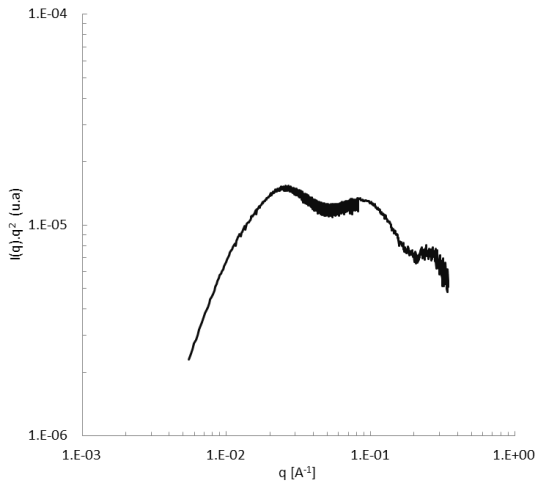

Figure II-6: Kratky-plot type representation of a gum arabic solution at 0.5% in 20mM NaCl by small angle x-ray scattering.

Structure of gum Arabic fractions probed by SANS and SAXS

Since gum Arabic is a heterogeneous mixture in size and hydrophobicity, one may question the relevance of scattering analysis on such systems. Indeed, the scattering spectra are a sum over all scattering parts, which potentially possess different structures, concentrations and contrasts. It seems thus preferable to analyze separately the different components of the gum. In practice this is challenging since the gum is a continuum of sizes and hydrophobicity but separated fractions from chromatography can be analyzed. The SANS and SAXS spectra of SEC fractions A, B, C and D (Figure II-1A and B) have been collected and are reported in Figure II-7A. The spectra of the smallest fraction (FD) could not be acquired due to its limited amount and its low solubility. Figure II-7C displays the scattering spectra of HIC fractions obtained by Renard, Sanchez and coworkers [13], [27], [29], [30]. Our set of SEC fractions exhibit spectra similar as the whole to that of gum Arabic using SANS measurements. The low-*q* range intensity of the largest size fraction, fraction A, is slightly higher than that of the other fractions and of the gum, suggesting it contains more parts responsible for this behavior than the two other fractions displayed. SAXS measurements on gum Arabic SEC fractions display a similar behavior as measurements of the whole gum Arabic in the intermediary and high q ranges. However, in the low q region, fraction A exhibits a higher intensity.

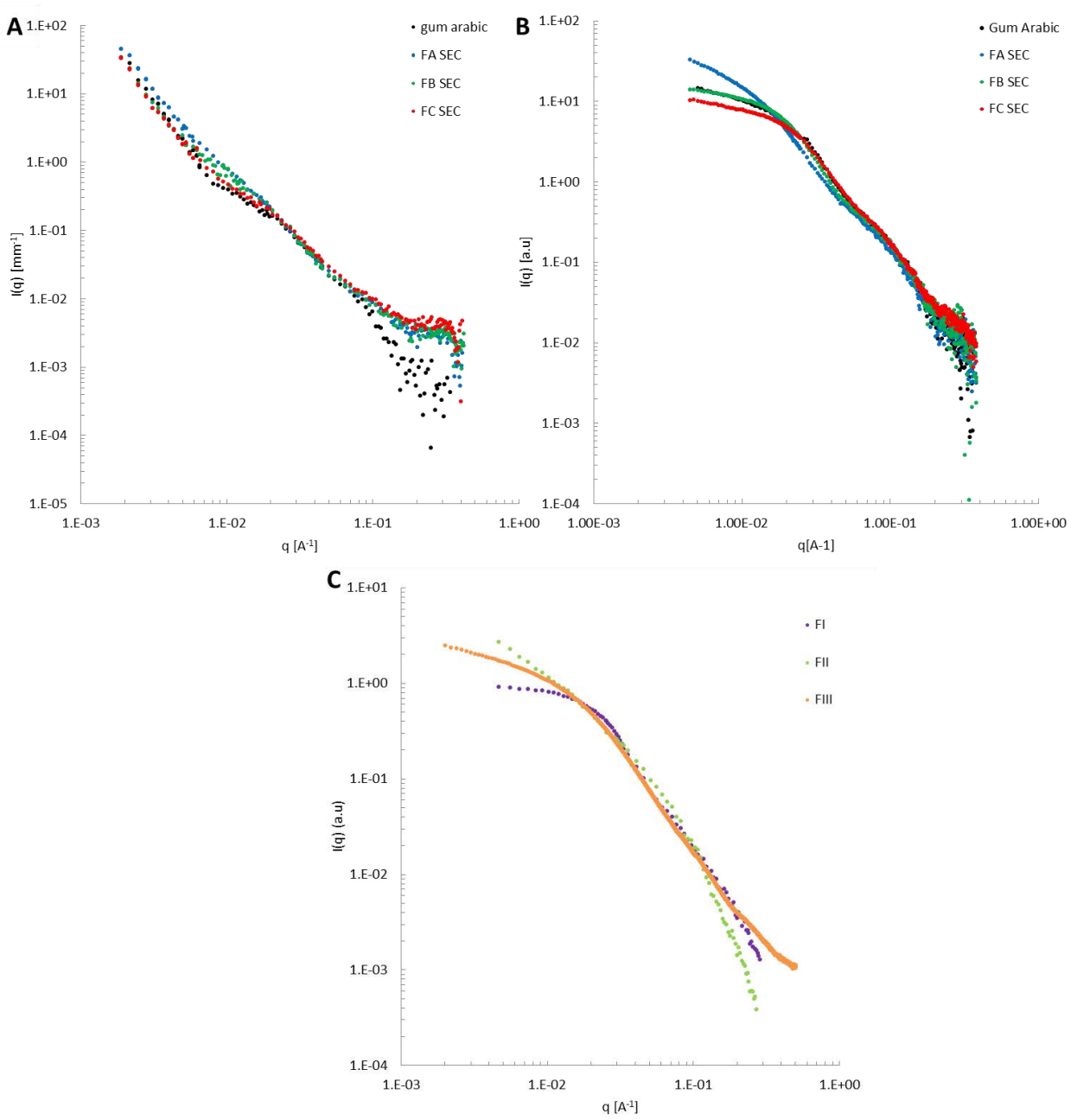

Figure II-7: (A) Small angle neutron scattering spectra and (B) Small angle x-ray scattering spectra of gum Arabic fractions separated by preparative size exclusion chromatography (FA represents the higher molecular weight and FC the smaller) measured at 0.5w/w% in 20mM NaCl D_2O solution (C) Small angle neutron (for FI and FII) or X-ray (for FIII) scattering spectra of gum Arabic fractions separated by preparative hydrophobic interaction chromatography from Renard, Sanchez et al., [27], [29], [30]. FI and FII were measured at 1w/w% in 50mM NaCl D_2O solution. FIII was measured at 1.7w/w% in 10mM acetate buffer pH5 and 100mM NaCl in distilled water. FIII intensity spectrum was rescaled to match that of the other fractions.

This overall similarity between the fractions spectra is not trivial since it could be expected that species of different sizes would display different structures and also that the smallest species would display a

finite size, which would be visible at a constant scattered intensity in the low-*q* range, which is observed in the SAXS spectra but not using neutrons. The similarity in the scattering curves suggests that the various species of gum Arabic are structurally related and that size is not an effective information to discriminate between structures.

Renard, Sanchez and coworkers rather characterized HIC fractions using either SANS or SAXS. Unfortunately, the low-*q* range was not investigated in their SANS experiments. Still, differences between the signature of each fractions are informative. In particular, the least hydrophobic fraction FI, clearly corresponds to a finite size object (Guinier range). Using the $Iq^2(q)$ plot, a radius of gyration radius of 6.2 nm can be estimated. This value is very similar to that of finite objects observed using SAXS on the whole gum, and the two objects are probably identical. For the two other fractions, the intensity does not reach a constant value in the probed *q*-range. It is likely that measurements at lower *q* values would have yielded the intensity upturn we observed in SANS measurements. In the intermediate-*q* range, all fractions seem to indicate a similar power decaying law scaling with exponents ranging between -2 and -2.2. The high-*q* behavior exhibits different power decay exponents smaller and larger than 2 in absolute value. Interestingly, oscillations in this *q*-range are also present with the most hydrophobic fraction (FIII measured using SAXS contrarily to FI and FII), and possibly with the intermediate hydrophobicity fraction. These oscillations are observed in each fraction we separated through Size exclusion chromatography using SAXS. The signal/noise ratio is too low in the SANS spectra to assess whether these oscillations are present or not. These oscillations could arise from several structural features: (i) several scattering populations (ii) a single scattering population displaying a multi-scale structure (iii) a single scattering population possessing a complex shape.

Discussion.

Two-dimensional chromatography shows that neither size nor hydrophobicity can be considered as truly efficient separation criteria. In this respect, some caution should be taken regarding the nomenclature used in the literature to distinguish different species (AGp, AGP, GP). Indeed, this terminology was used for both size or hydrophobicity separations, which are not equivalent. However, the two-dimensional analysis allows to disentangle rather homogeneous populations, which would be the basic elements from which the various species are made of. We will first describe these populations before turning to structural schemes of gum Arabic in solution.

The Arabinogalactan-peptide population as the building block of gum Arabic

Both our SEC/HIC chromatogram and the HIC/SEC chromatogram of Renard and coworkers indicate that a rather homogeneous population of intermediate size species exists, corresponding to the lowest hydrophobicity [13]. This population was shown to remain intact during enzymatic attacks of polypeptide chains and displays the minimal protein content amongst the gum species. As pointed out by others [1], this population shares similarities with arabinogalactan peptides of other plants and the terminology AGp seems indeed adequate. Structurally, this population is the only one that corresponds to finite size objects over the length scales investigated, with a radius of gyration of 7 nm and a spherical radius of 9 nm, matching previous measurements of hydrodynamic radius [30]. This population comprises charged moieties, uronic acids, which give rise to a structure correlation peak at low ionic strength. This population dominates both SANS and SAXS scattering spectra of gum Arabic in the intermediate length scale 5-15 nm. At lower length scales, the behavior seems somehow similar to that of gum Arabic or other fractions. Moreover, oscillations are as visible in the gum Arabic SAXS spectra as well as in the fractions separated through size exclusion chromatography. Sanchez and coworkers proposed a flat disk model for AGp based on the value of the spectrum slope and on various surface imaging techniques (TEM, Cryo TEM, AFM). However, a porous sphere corresponding to a mass fractal of fractal dimension 2 may equally describe the scattering curves. Interestingly, many other power laws deviating from the value -2 are also observed with the different fractions or even within a given spectrum depending on the precise considered *q*-range. One may argue for a variable porous inner structure of a flat ellipsoid but this means that such an object is porous, which rises the question of the higher probability of a two-dimensional structure against a three-dimensional one. A few arguments seem to rather support the three-dimensional porous structure. First, hydrodynamic radii match exactly SAXS and SANS radii estimated from the radius of gyration of a spherical shape, while a 50% discrepancy was reported by Sanchez et al. with the flat disk model. Secondly, the gum concentration-dependent signature of the structure peak of gum Arabic, which stems from ionic repulsions, is consistent with a population of repulsive objects up to a 5 w/w% concentration, with a shift of the peak position to higher *q* values and an increase of the peak height as the gum concentration is increased. However, further increase of the concentration leads to a broadening and decrease in peak height [26]. This observation suggests that objects interpenetrate and reorganize, which seems difficult to understand with a two-dimensional structure. Furthermore, the 15 to 20 nm distance at overlap is more consistent with a spherical objects dimensions. Last, both SANS and SAXS spectra yield similar form factors for these objects despite differences in the low-*q* range, which were attributed to hydrogen-deuterium exchanges, that caused a

highlighting of the polypeptide backbone. Therefore, the polypeptide backbone of AGp must possess a similar structure than that of the whole of the scattering parts of the gum to remain the same despite differential contrast changes.

Regardless, it has been suggested by Mahendran et al. that the AGp fraction would actually be composed of much smaller blocks than what is suggested by the chromatographic analysis [20]. Their demonstration rests on the analysis of size exclusion chromatograms of gum Arabic, before and after a mild reducing attack, which cleaves mainly O-serine bonds. They stated in their paper that the 1-5.10^5g.mol^{-1} population corresponding to the AGp moieties, remained unchanged while the 2.10^6g.mol^{-1} fraction, that they attribute to AGP conjugates, disappeared to yield a 4-5.10^4g.mol^{-1} peak. This led them to conclude that polysaccharide-rich blocks from AGP are much smaller than those of AGp. However, in their data, the formation of the 5.10^4g.mol^{-1} peak and the disappearance of the AGP peak are concomitant with a an increase of the AGp peak. It is thus more likely that AGP are indeed made of AGp blocks, which are linked by other species that are not attacked in this mild reducing treatment, but attacked in the enzymatic treatment. Therefore, the data from Mahendran et al. can rather be interpreted as a direct proof that AGP are made of AGp blocks linked by polypeptide chains. We shall return to this point later.

Hidden in plain view: a second population of slightly amphiphilic AGp as building block

From our two-dimensional chromatogram, another rather singular population can be identified. It corresponds to intermediate sizes and is not distinguishable from the main peak in the refractive index detection but corresponds to a clear peak in UV absorption at 210 and 280 nm (Fγ: 135kg.mol^{-1}, and FC). Since it is much more intense with respect to other fractions at 210 nm than at 280 nm, we can deduce it contains less hydrophobic amino-acids (tryptophan and tyrosine) than average. Also, its hydrophobicity and protein content are slightly higher than the main population of AGp, leading to identify it as a separate population. This second AGP population is much less concentrated than the main population but is at least as numerous owning to its smaller size. Interestingly, Mahendran et al. actually observed that enzymatic hydrolysis of gum Arabic, which impacts the large Arabinogalactan-protein conjugates, yielded both the large AGp population described in the previous section but also this smaller population (Figure 4 of [20]). We can thus deduce that AGP conjugates are actually build from a mixture of these two AGp building blocks.

The SAXS analysis of FC, corresponding to the second population of AGp, also revealed the presence of oscillations in the intermediary q range. Moreover, circular dischroism measurements were performed on gum Arabic and the gum SEC fractions, in order to get more information about the secondary structures of the gum species. Results are presented in Figure III-14 in supporting information. It was observed that only FA presents an optical response similar to that of the whole gum Arabic whereas fractions FB and FC do not present an optic activity. This observation seems to indicate that the fractions corresponding to the two populations of AGp do not present species with a secondary structure. Nevertheless, since oscillations are observed for all gum fractions in SAXS spectra, we can deduce they are unrelated to an inner multi-scale structure of a scatterer. The hypothesis of a several scattering population is the only remaining one and a likely one since we have observed several populations in gum Arabic. The second oscillation in the intermediary q range could thus be the signature of this second population of AGp.

Polypeptide chains link the building blocks

We have observed a striking difference between SAXS and SANS spectra at large length scales that we attributed to a highlighting of polypeptide chains in SANS experiments. Since we probed length scales up to 300 nm without any signs of reaching a finite size, it is likely that the polypeptide network is composed of several chains. This would be consistent with the rheological behavior of gum Arabic and light scattering observations of self-association [25], [31], [34], [35]. Two power law behaviors are observed depending on gum concentration. At low gum concentration (0.5% with salt), for decreasing (resp. increasing) q (resp. length scales), we first observe an increase in q^{-1} in the intermediate-q range, followed by an increase in q^{-3} (Figure II-4). At higher gum concentration (5% no salt), we observe a more extended increase in q^{-2} (Figure II-4). Therefore, increasing the gum concentration leads to an increase of the compactness in the range 10-100 nm but a decrease of compactness decrease in the range 100-300 nm. The low concentration behavior would be then consistent with collapsed networks build from rigid blocks, which would swell upon increasing their concentrations. The driving force must originate rather from polypeptide/solvent interactions rather than ionic repulsions since the slopes were unchanged upon screening the structure correlation peak. We can thus conclude that polypeptide chains structure solutions of gum Arabic at large length scales. These polypeptide chains are ubiquitous in gum Arabic, and only the less hydrophobic fraction collected in HIC chromatography does not seem to contain it.

Glycoproteins, film formation and hydrophobicity

As discussed above, enzymatic degradation of both gum Arabic or gum Arabic intermediate HIC fraction show that the largest population of gum Arabic degrades enzymatically into a population of similar size as the two AGp population. A major difference in the amount of secondary structures probed by circular dichroism is however observed. Interestingly, a mild reducing attack as performed by Mahendran et al., leads to the increase of the AGp peak, contrarily to what was stated in the paper, and a peak around 4-5.10^4 $g.mol^{-1}$ [20]. This last peak corresponds in size to the highly proteinaceous fraction of gum Arabic as we can see on our chromatograms (Fraction D). This is consistent with this population being absent after enzymatic treatment since such a treatment is expected to cut accessible polypeptide chains, while it is expected to remain in the mild reducing treatment that mainly attacks o-linkages between peptides and sugars. Size exclusion chromatograms at 210 nm display two distinct populations, while at 280 nm four distinct peaks are visible (Fraction δ in Figure II-1A and 1B after 11mL of elution). The large differences between the 210 and 280 nm chromatograms in terms of peak intensities demonstrate that these polypeptide rich populations are highly heterogeneous in their amino-acid content. Rather than referring to a glycoprotein population, these observations indicate that several glycoprotein populations exist with different backbones. This casts some doubts about the generality of both palindromic pattern for gum Arabic proposed by Goodrum et al. and the two bands obtained after HF deglycosylation by Mahendran et al. [18], [20]. Importantly, this fraction (Fδ) behaved in an original fashion after being collected through SEC chromatography and freeze-drying. While the other fractions yielded a cloud-like powder after freeze-drying, this fraction was collected as a thin film, which could not be properly solubilized in water. This suggests a strong aggregation in concentrated conditions, which was also observed in scattering curves of this fraction. This aggregation behavior suggests that glycoproteins backbones are at the core of amphiphilic properties of gum Arabic and partly correlated to their content in hydrophobic amino-acids.

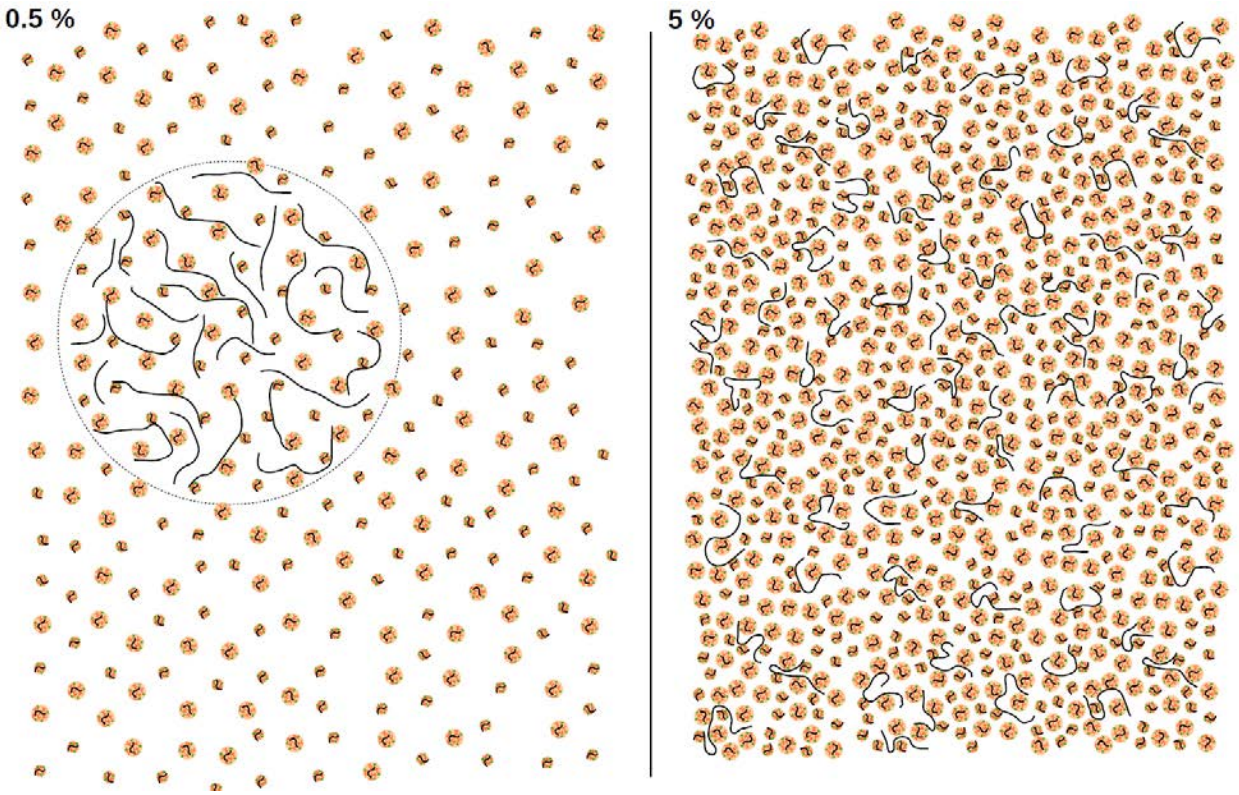

Figure II-8: True scale schematic representation of gum Arabic macromolecules in solution as a function of concentration. Black segments represent the glycoproteins moieties while the globular elements represent the arabinogalactan-peptide (AGp) (two sizes for the two populations). Segments linked with AGp represent the arabinogalactan-protein conjugates (AGP). The scheme respects the multiscale structure of gum Arabic species in solution for two concentrations: dilute at 0.5% and close to overlap of AGp at 5%. The scale is given by the correlation distances extracted from structure peak positions at low ionic strength.

Conclusion: Gum Arabic in solution as a multiscale structure.

From experimental results described in this work and many results previously published, we have arrived at some important clarifications regarding previous structural models of gum Arabic. We have shown that two distinct populations of arabinogalactan-peptides (AGp) in similar numbers, with the less concentrated one corresponding to smaller sizes but larger protein content and hydrophobicity despite a rather weak content of hydrophobic amino-acids. Several glycoproteins are present and display important differences in their content of hydrophobic amino-acid and sizes. Despite their size and concentration, they are responsible of the large length scales structures (at least 300 nm) that develop in solution and they are prone to aggregation. These structures are visible in gum Arabic and in the large size SEC fraction and depend on gum concentration but not ionic strength. The larger species in gum Arabic are arabinogalactan-protein conjugates (AGP) as it has been extensively described in the literature, but we have evidenced that they are composed of the two types of AGp, which are attached

together to the glycoproteins. Sub-populations of AGP are thus present in gum Arabic. Figure II-8 displays a true scale scheme of the multi-scale structure of the different gum Arabic species in aqueous solutions under two concentration regimes.

Materials and methods.

Materials

Powder gum arabic (Acacia senegal) was a gift from Caragum International ® (Marseille, France) and the composition was 2.9% of proteins (Nx6.25), 11% moisture, 3.16% ash and nearly no lipid. Sodium chloride (99.5>% BioXtra), hexadecane (99% ReagentPlus), pentane and hydrochloride acid were purchased from Sigma Aldrich. Distilled water was used for all experiments.

Electrophoresis

Electrophoretic mobility measurements were performed using a Zetasizer NanoZS instrument (Malvern Instrument) in DTS1060 cells. Solutions of 0.1w/w% of gum Arabic were measured and the pH was adjusted by either addition of HCl or NaOH. Measurements were performed at 25°C as mean of triplicates.

Size exclusion chromatography

Size exclusion chromatography was used to separate Arabic gum species as a function of their relative hydrodynamic volume. A 7.8mmx300mm BioSuite 450Å SEC column (Waters ®) packed with 8µm porous silica beads was used. The average pore size of the silica beads is 450Å.

The separation was performed on an Alliance HPLC unit (Waters 2695 separations module), a 0.5mol.L^{-1} NaCl aqueous solution at 25°C was used as the eluent phase at a flow rate of 0.8mL.min^{-1}. Each sample was filtrated with a nylon 0.2µm membrane and a volume of 50µL was injected. UV detection was performed at 210 nm and 280nm using a Waters® 2487 UV detector. A refractive index detection was used to measure the mass percentage of each eluted moity (Waters® 410 differential refractometer). The refractive index detection was only available until 10.5mL of elution due to a negative peak appearing from the difference in refractive index between the sodium chloride of the eluent phase and the water from the injected sample. The column was calibrated using branched dextran standards (Waters ®). [36]

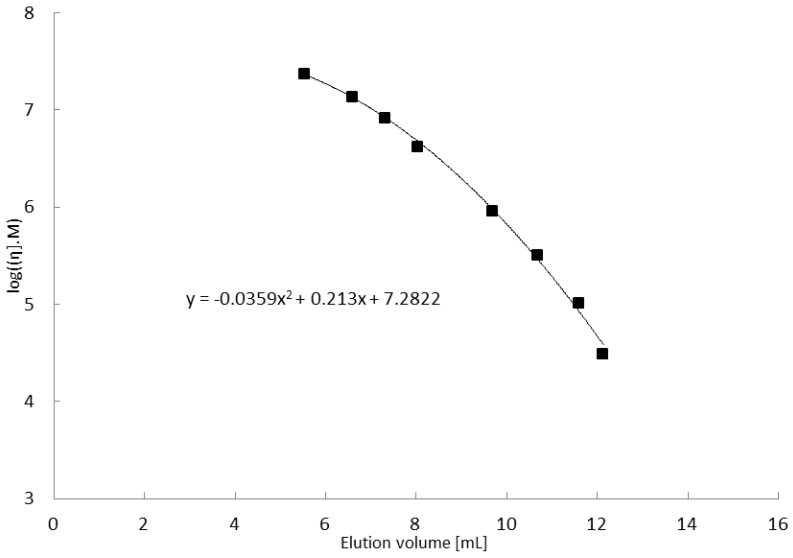

Figure II-9: Universal calibration curve (realized with branched dextran of known molecular weight) for size exclusion separation on Biosuite 450Å SEC column (waters) with a low rate of 0.8mL/min and 0.5M NaCl as the eluant solution.

Variation coefficients for each detection were calculated from three injections of the same sample. For UV detection at 210nm a 0.3% variation coefficient was measured. It was of 0.5% for UV detection at 280nm and of 13% for the refractive index detection.

UV detection at 280nm is more sensitive to the rate of cyclic amino acid present within the gum polypeptidic moieties (tyrosin and phenylalanine). Cyclic amino acids however are not the predominant amino acid moiety in gum Arabic, as already observed in the literature [12], [18], [24]. UV detection at 210 nm is mostly sensitive to $\pi \rightarrow \pi^*$ transitions from C=O bond of the amides linkages between amino acid moieties, and from those of carboxylic moieties present onto the polysaccharides backbones and of reducing sugar units at the end of polysaccharides chains.

Hydrophobic interaction chromatography

Hydrophobic interaction chromatography was used to separate Arabic gum fractions as a function of their hydrophobic properties. A 7.5mmx75mm 10µm Biosuite Phenyl column (Waters ®) which consist of phenyl groups bonded to a metacrylic ester based polymeric resin. The average pore size of the column is 1000Å to accommodate macromolecules with high molecular weight. A gradient of salt concentration is required to create a “salting out” effect and progressively desorb the hydrophobic species. The less hydrophobic species are eluted first with highest salt concentration, while the more hydrophobic species are the last one eluted.

The separation was performed on an Alliance HPLC unit (Waters 2695 ® separations module). The mobile phase was composed of a solution of NaCl at a constant flowrate of 0.5mL.min^{-1}. A continuous time gradient of salt concentration between 4mol.L^{-1} and 0 was applied during 22minutes followed by distilled water during 12 more minutes (Figure II-10). Each sample was filtrated with a nylon 0.2µm membrane and a volume of 30µL was injected. UV absorbance was measured at 210 nm and 280nm (Waters® 2487 UV detector). We observed that UV absorbance (especially at 210nm) was very sensitive to NaCl concentration. The baseline was not constant along the separation time. In order to correct this deviation, the chromatogram of distilled water injected in same elution conditions was subtracted to each measured chromatogram.

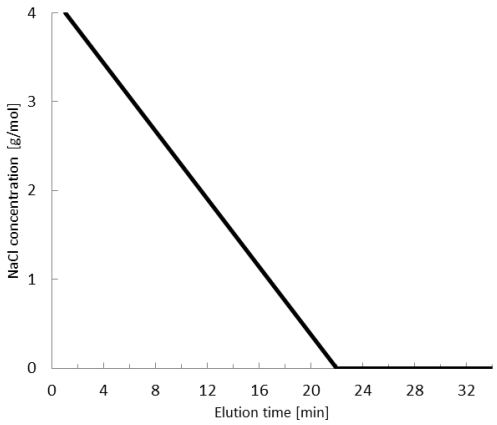

Figure II-10: NaCl elution gradient used for hydrophobic interaction chromatographic separation.

Variation coefficients for each detection were estimated from comparison of three injections of the same sample. A 6% (resp. 11%) variation coefficient was observed at 210nm (resp. 280nm).

Two dimensional chromatographic separation

Five fractions were collected using size exclusion chromatography as described before. A volume of 80µL of a gum solution at 5w/w% was injected in the SEC column. Two separations were carried out and fractions of 120µL were collected and freeze-dried. These fractions were then dissolved in 50µL of distillated water and 20µL of each was injected in the HIC column for the second dimension separation. Samples concentration being relatively low only UV detection at 210nm was available.

Small angle neutron scattering (SANS) experiments and data treatment

SANS experiments were performed on the PAXY small angle diffractometer located at the Orphee reactor in Saclay, France. Samples were dissolved in 20mM NaCl in D_2O solution. Spectra were recorded

using four different spectrometer configurations: λ=6Å (incident wavelength), d=1m (distance of the sample to the detector), λ=6Å and d=3m, λ=8.5Å and d=5m and λ=15Å and d=6.7m. The range of wave vectors *q* covered extends between $6.5.10^{-3}$ and $4.5.10^{-2}$ $Å^{-1}$. The data were normalized for transmission and sample path length. A subtraction of the incoherent background was performed using the sample transmission at high q values and a calibration of D_2O/H_2O solutions at different ratios (transmission as a function of scattered intensity).

Small angle X-ray scattering (SAXS) experiments and data treatment

SAXS experiments were performed on the ID02 instrument at the ESRF synchrotron facility in Grenoble, France. Samples were prepared using 1.5 mm disposable quartz capillaries. Measurements were taken at 1.5 and 7m. Azimuthal averaging was performed after mask subtraction and data was normalized by the transmission. One dimensional spectra were subtracted by the capillary filed with water or the buffer solution.

Circular dichroism

Circular dichroism measurements were performed on gum Arabic and gum SEC fraction on a Jasco J-815 CD spectrometer at IMRCP laboratory in Toulouse, France. A quartz cell with a 10mm path length was used. Measurements were performed between 250 and 185nm at 25°C. A concentration of 0.025% was used for gum Arabic and of 0.00625% for FA, FB and FC. FD was not measured due to its low solubility. Results were normalized by the concentration and the path length to be comparable.

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Supporting information.

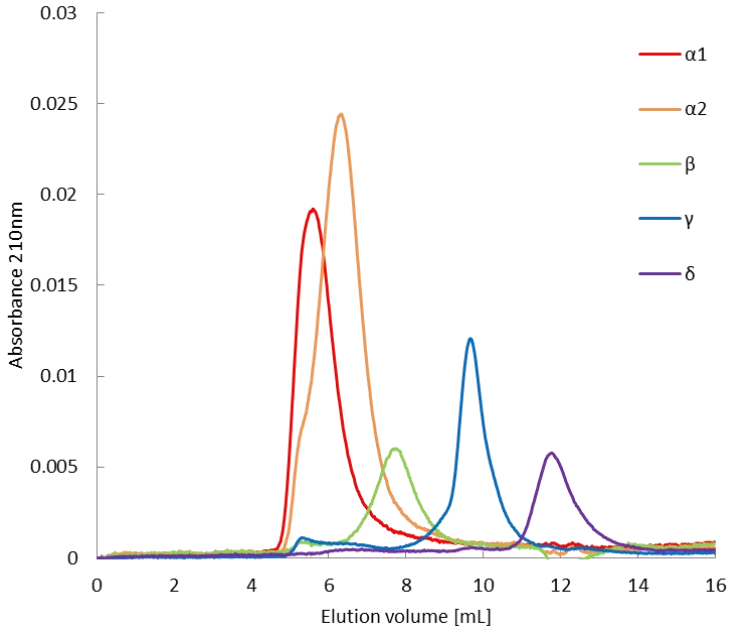

Figure III-11: Control of collected fractions by size exclusion chromatography with a UV detection at 210nm. Concentration for each fraction was relatively low (low UV absorbance) and did not permit to use the refractive index detection.

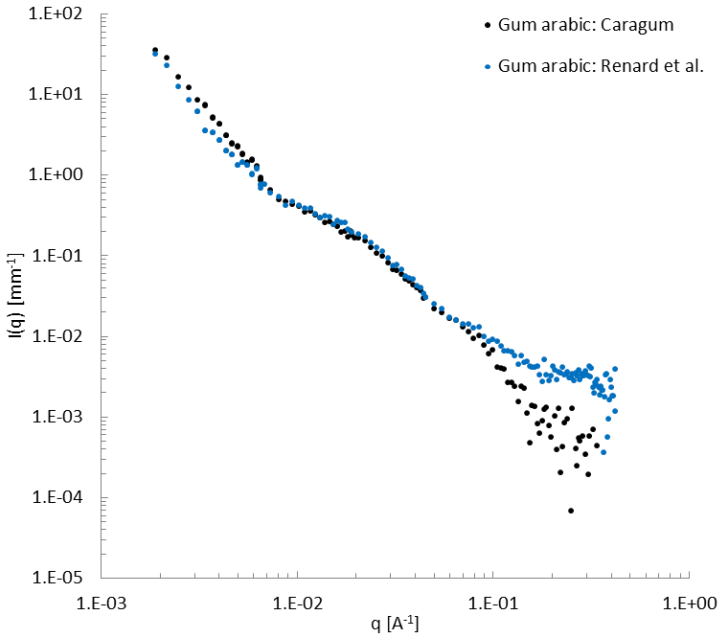

Figure II-12: Small angle neutron scattering spectra of our gum sample (Caragum) and Renard et al. gum sample. Sample were measured at 0.5w/w% gum concentration with 20mM NaCl in D_2O.

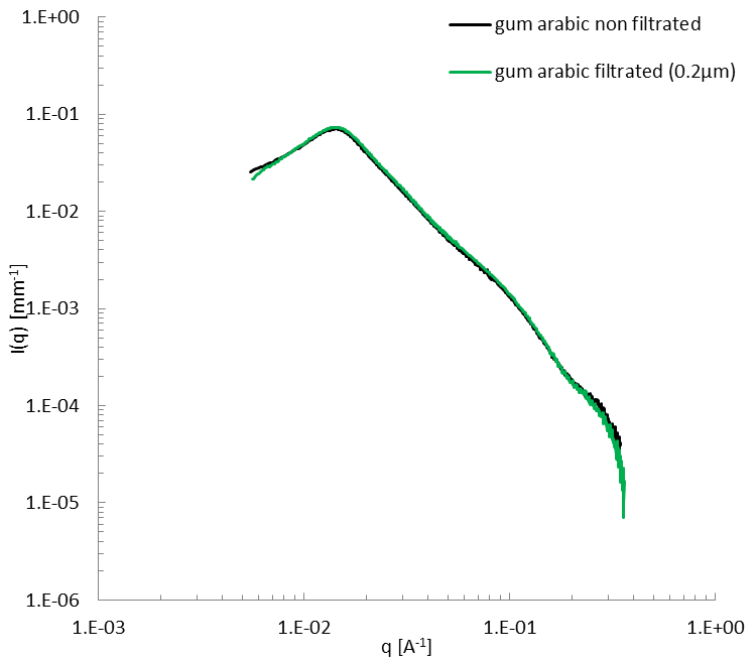

Figure III-13: Small angle neutron scattering spectra of gum Arabic solution with or without filtration. Samples were measured at 0.5w/w% gum concentration in D_2O. The perfect match of both spectra indicates that filtration has no impact on the scattering spectrum.

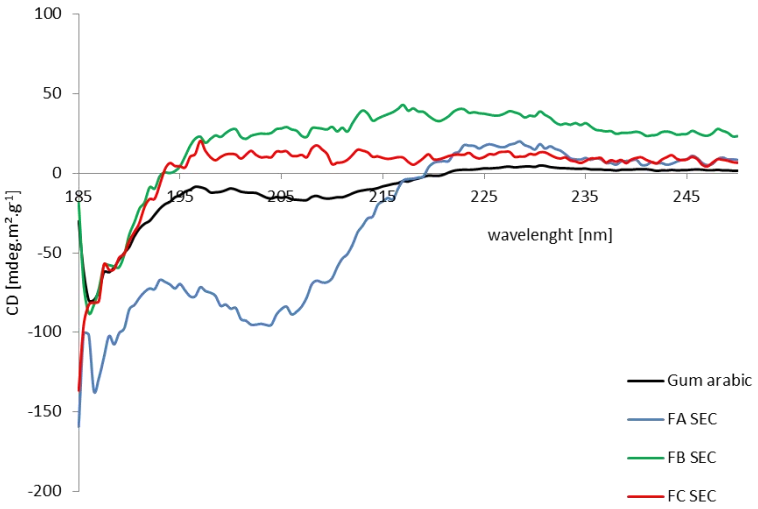

Figure III-14: Circular dichroism measurements of Arabic gum and gum fractions separated by preparative size exclusion chromatography (FA represents the higher molecular weight and FC the smaller).

Electrophoretic mobility

We decided to perform electrophoresis measurements on gum Arabic aqueous solution as a function of the solution pH. The gum being composed of polyelectrolytes (from the carboxylic moieties on glucuronic acid polysaccharide parts) we assumed its global charge to be dependent on the solution pH.

From the literature the pKa of glucuronic acid is around 3.2 [37], [38]. At pH=pKa 50% of the carboxylic moieties are deprotonated. Thus at the isoelectric point all the carboxylic function are protonated. Therefore at pH 3.2 the electrophoretic mobility should be around 50% of its value when carboxylic moieties are completely deprotonated (pH values above pKa).

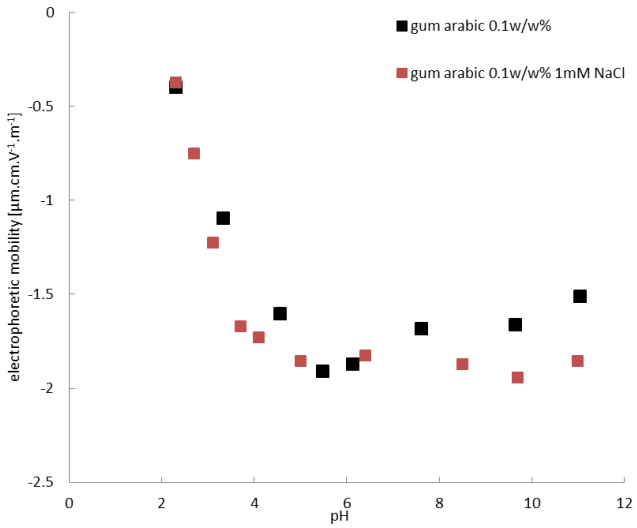

Figure III-15: Evolution of gum arabic electrophoretic mobility in aqueous solution as a function of solution pH.

Results obtained are similar to one obtained by Padala et al. [23]. The electrophoretic mobility is negative over all the pH scale. Gum Arabic polyelectrolytes are thus negatively charged. Above pH 6 the electrophoretic mobility appears to be constant (in the presence of added salt) around -1.9 µm.cm.V^{-1}.m^{-1} and start increasing while reducing the solution pH. The isoelectric point of the gum can extrapolated around pH=2. At pH=3.2, the electrophoretic mobility seems to be around 2 two which approximately correspond to a reduction of 50% of the charged which is consistant with a pKa of 3.2. A variation of gum Arabic solutions pH can therefore influence its charge and possibly its behavior in solution and at the interface.

Gum Arabic solubilisation

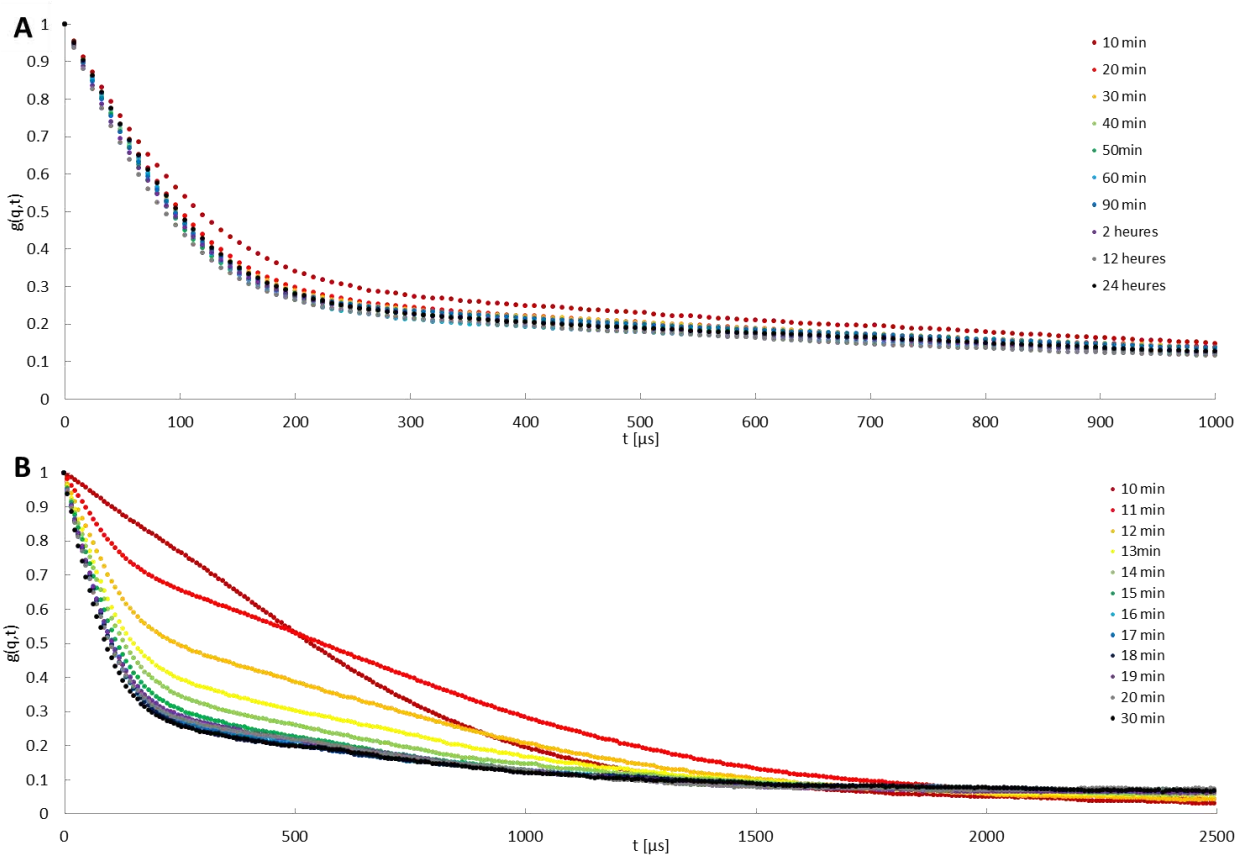

Figure III-16: Evolutions of 3 w/w% gum arabic solutions dynamic light scattering auto-correlation functions as function of agitation time (A) over 24h and (B) over 30 min. The gum species appear to be completely solubilized after 30minutes of agitation (the auto-correlation functions are stable).

Chapter III- Gum Arabic stabilized emulsions: composition of the interfacial film

Abstract.

Gum Arabic is a heterogeneous natural hydrocolloid used notably in agro-food industry to provide metastability to oil-in-water emulsions. Since aqueous solutions of gum Arabic contain a complex mixture of protein/polysaccharides conjugates, the composition of interfacial films is expected to differ from the bulk composition. Here, we investigate the composition of interfacial films in oil/water emulsions stabilized by gum Arabic under various concentration regimes and physico-chemical parameters. Using both size exclusion and hydrophobic interaction chromatography separations, we show that the interface is enriched in protein rich species displaying a broad range of sizes. These species are irreversibly adsorbed as monolayers at the oil/water interface. We observe that the surface density of adsorbed species at oil/water interfaces drastically increases with both increasing gum concentration and decreasing ionic repulsions through increasing ionic strength or decreasing pH. Strikingly, these surface density changes correspond to only minor composition changes in the adsorbed layer. We thus conclude that the key parameter modified in different formulations is the conformation of adsorbed species rather than their composition distribution.

Introduction.

Gum Arabic is an exudate originating from acacia trees in sub-Saharan countries, which has been used for centuries for its binding and stabilizing properties [1]. Nowadays, it is mainly used to stabilize dispersed systems such as oil-in-water emulsions, in particular to formulate flavored beverages [2].

This natural hydrocolloid, highly soluble in water, is composed of a complex mixture of bio-polymers. Gum Arabic structural composition has been extensively studied in the literature using chromatographic separation techniques such as size exclusion chromatography or hydrophobic interaction chromatography. First, separation of gum species depending on their relative hydrodynamic radius (molecular weight) has revealed the heterogeneous nature of the gum, identified as a mixture of proteins and polysaccharides moieties covalently linked with a mean molecular weight around $5.10^5 g.mol^{-1}$[3], [4]. Size exclusion chromatography has shown the presence of a high molecular weight fraction associated to the gum polypeptide moieties [3]. Hydrophobic interaction chromatography on gum Arabic exhibit three fractions differing in hydrophobicity (related to their protein rate) [5]–[10]. These are described as an arabinogalactan rich polysaccharide fraction constituting the bulk of the gum (AG) (88%), a polysaccharide-protein conjugates fraction (AGP) (10%) and a fraction of glycoproteins (GP) (2%). The conjugates fraction has been identified as the main responsible for the emulsifying properties of the gum with the protein part adsorbing at oil droplets surface and the carbohydrate moieties providing steric repulsion between droplets [3]. The

use of Yariv reagent on these three fractions has confirmed the presence of conjugated moieties in each of them [7]. Several studies have been carried out to analyze the structure of the gum protein-polysaccharides species. The first model described the gum as a wattle blossom shape with a peptide chain linked to fives branched carbohydrate moieties [3]. Then a model describing the gum as a twisted hairy rope was proposed [11]. More recently, small angle scattering experiments have been performed to probe the structure of the gum fraction recovered from hydrophobic interaction chromatography and the AGP fraction was modeled as thin ellipsoids [12]. In a previous work the macromolecular composition and structure of the gum was studied using small angle scattering experiments (neutrons and x-ray). Gum Arabic in solution was described as a multiscale structure with two populations of arabinogalactan-peptide (AGp) differing in sizes and protein content and several glycoproteins with a variable content of hydrophobic amino-acids. Conjugates of these different species were also present and correspond to large arabinogalactan-protein conjugates (AGP).

If quite a number of studies have been conducted to improve the understanding of the gum species structure in solution, only few have been devoted to the actual composition of adsorbed gum Arabic films. Randall et al. analyzed the aqueous phase from a gum stabilized emulsion by size exclusion and observed that the high molecular weight macromolecules preferentially adsorbed at the interface. Using the same procedure Padala et al showed that a percentage of each classes of the molecular weight spectrum was adsorbed [3], [13]. Emulsifications using recovered gum Arabic fractions from size exclusion chromatography (size) or hydrophobic interaction (protein content) showed that using a combination of both small and the high molecular weight species provided the best emulsion metastability [14]. Studies have been achieved to evaluate the surface concentration of gum-stabilized droplets but without evaluating the composition of the film. It was observed that physico-chemical parameters such as the pH or the salinity promoted the interfacial adsorption [13], [15], [16], but no systematic study was performed to evaluate the composition changes at oil/water interfaces.

In the present work, we present a method to systematically characterize the interfacial composition in molecular weight and hydrophobicity as compared to that of the bulk. Surface coverages of gum Arabic-stabilized emulsions as a function of the emulsion formulation (gum concentration and physico-chemical parameters) are compared. Finally, compositions of the interface as a function of these parameters are discussed.

Results and discussion.

Chromatographic characterization of gum Arabic solutions

Gum Arabic is a mixture of polysaccharides/protein species, which displays a continuum of sizes and hydrophobicities [4], [5], [17]. Since gum Arabic is a natural product, some composition variabilities are expected with its origin [8], [18]–[20]. Both size exclusion and hydrophobic interaction chromatography techniques have been used in past studies on gum Arabic. We have thus used these two techniques to characterize the batch of gum Arabic used throughout this work.

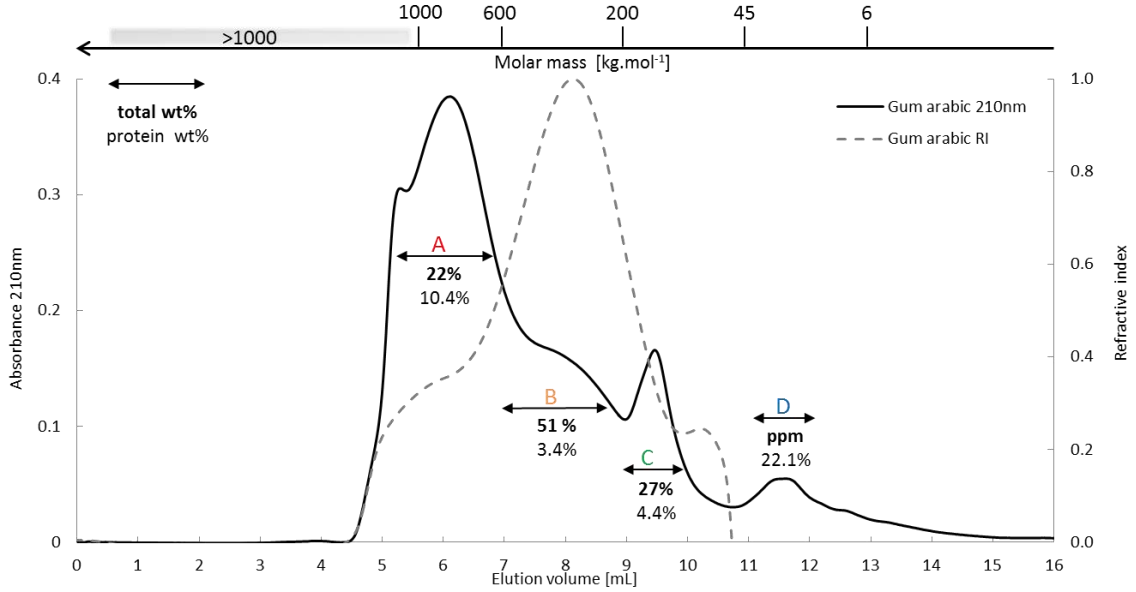

Figure III-1: Size exclusion chromatogram of a 3w/w% aqueous solution of gum Arabic (injection volume: 50µL). Thick line: UV detection at 210nm. Dotted line: Refractive index detection. Weight percentages of recovered fraction using a preparative size exclusion column (same column filling, larger dimensions) are indicated as well as the protein rate for these fractions. Scale for molecular weight as a function of elution volumes is displayed on the top of the chromatograms and was obtained using branched dextrans of known molecular weight (universal calibration curve). (SEC column: Biosuite 450A SEC Waters ®)

Figure 1 displays the size exclusion chromatogram of our gum Arabic sample (Caragum International ®) using both a UV detection at 210 nm, which is mostly sensitive to the peptide bond [21], [22], and a refractive index detection, which is proportional to concentration. This chromatogram is similar to previously published gum Arabic chromatograms [19], [17], [13], which ensures the representativity of our gum Arabic batch. We observe that gum Arabic displays a broad range of molecular weight ranging from 6 $kg.mol^{-1}$ to more than 1000 $kg.mol^{-1}$. From the refractive index detection, we observed that gum is majoritarily composed of the intermediary molecular masses, whilst the higher molecular masses (peak 1 and 2) represent around 15 wt% of the gum. This is consistent with the weight fractions of the different gum Arabic fractions obtained through a similar preparative chromatographic separation (A, B , C and D from

Figure III-1). The protein weight fractions were also determined and display variations ranging from 3.4 to 22.1% between the different fractions. In a precedent study we showed that the first peak (higher molecular weight) correspond to the gum arabinogalactan-protein conjugates (AGP), the

second and third population (shoulder and peak at 9.5mL) correspond to two populations of arabinogalactan-peptide (AGp) and finally the peak at 11,5mL correspond to the gum glycoproteins.

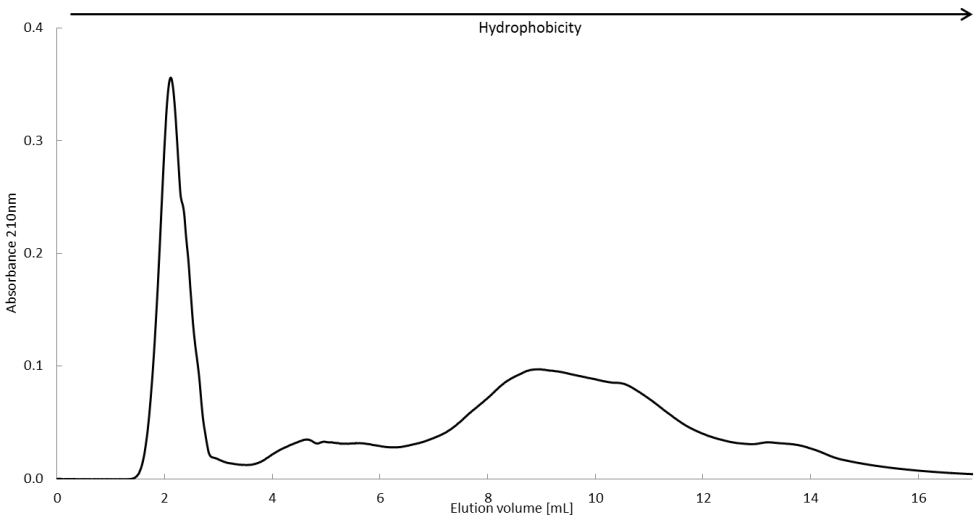

Figure III-2: Hydrophobic interaction chromatogram of gum arabic. UV detection at 210nm. Less hydrophobic species of the gum are not retain by the column and species eluted last are the more hydrophobic ones as indicated by the scale on top of the chromatogram. (HIC column: Biosuite Phenyl HIC Waters ®)

The protein content measured in the four fractions (from size exclusion separation: A, B, C and D) suggests that the various species of gum Arabic may display amphiphilicity. We have then used hydrophobic interaction chromatography to probe the hydrophobicity of gum Arabic, which corresponds to its interaction with a phenyl grafted column. Unlike previous works [5], [14], [17], a continuous gradient of sodium chloride concentration was imposed along the column height and the resulting chromatogram is displayed in Figure 2. The first peak corresponds to the void volume of the column and hence to species that are not retained by the column. This intense and narrow peak indicates the presence of a rather homogeneous population of hydrophilic species in gum Arabic. In contrast, the rest of the chromatogram displays a broad elution profile, which emphasizing the heterogeneity of the gum as a continuum of species of increasing hydrophobicities. The dual chromatographic characterization of gum Arabic highlights its complex composition in size and hydrophobicity and thus calls for further investigation of the interfacial composition of oil/water interfaces in the presence of gum Arabic.

Is adsorption of gum Arabic species an equilibrium process?

When an aqueous solution of amphiphilic species is placed in contact with an oil phase, some species are expected to adsorb at the interface. This adsorption may proceed along a thermodynamic pathway and is then controlled by adsorption equilibrium constants, which yields experimentally adsorption isotherms. This is typically the case for short chains surfactant molecules [23]. Adsorption

may alternatively proceed through a kinetic pathway, which will depend on both adsorption equilibrium constants and transport properties during the interface formation. This corresponds for instance to the case of particle-stabilized emulsions. In both cases, the interfacial composition is expected to differ from the bulk composition when species of different amphiphilicities and sizes are present in water. However, distinguishing between thermodynamically controlled or kinetically controlled adsorption is crucial to determine experimentally the interfacial composition. To this purpose we prepared an oil/water emulsion, which was centrifuged in order to collect a concentrated cream. We then rinsed several times this cream and measured after each rinsing step the amount of gum Arabic species taken away from the cream (Figure III-3). Around 1.4% of the initial gum amount is collected after the first rinsing, which corresponds to species that were trapped in the aqueous phase in between oil droplets. Subsequent rinsing yields negligible quantities of gum Arabic. Yet the resulting cream displays an even higher metastability than the original emulsion, demonstrating that adsorbed species are not leached out by dilution as would be expected for an equilibrium adsorption process. Removing both water and oil from the rinsed emulsion yields the total mass of gum Arabic adsorbed, which corresponds to 15.8% of the total gum Arabic before rinsing and to a constant value of 13.8% after the second rinsing. We can thus conclude that oil/water interfaces of gum Arabic stabilized emulsions are mostly populated by irreversibly adsorbed species. This property is routinely used in the beverages industry, where Arabic gum is used to stabilize concentrated aroma oil/water emulsions that will be highly diluted in the final beverage.

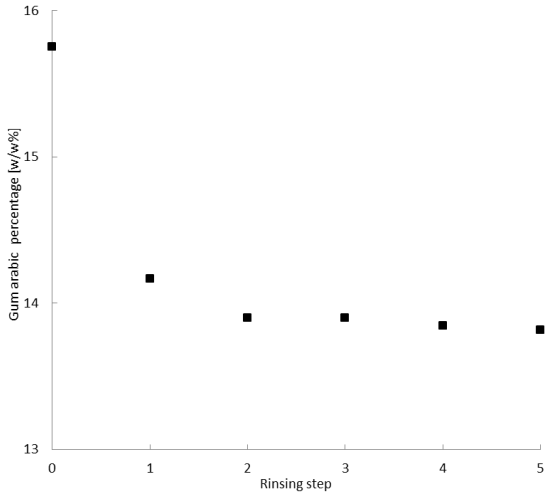

Figure III-3: Evolution of gum Arabic mass percentage in the emulsion cream (compared to the amount of gum in the emulsion 100% is the amount used in the emulsion formulation) when rinsing an emulsion cream as a function of the number of rinsing steps. 0 correspond to the amount of gum in the emulsion cream without rinsing.

However, we also observed that upon intense centrifugation conditions droplet coalescence could occur, leading to a partial desorption of some species. Reducing the interfacial area may thus force

some species to desorb, which is consistent with microscopy observations showing that emulsion droplets remain spherical. From a thermodynamic standpoint, this means that the adsorption energy of some adsorbed species is smaller than the gain in relaxing droplet's shape upon coalescence. The average area occupied by gum species is thus bound by a maximal value.

How does the films composition compare with the bulk composition?

Since adsorption of gum Arabic species can be considered as irreversible, the interfacial composition will be preserved upon dilution. Taking advantage of this property, we have designed an experimental method to determine the interfacial composition of gum Arabic stabilized emulsions. From a given emulsion the non adsorbing species were collected by recovering the emulsion aqueous phase. The remaining emulsion cream was rinsed several times to recover all species still potentially present in the aqueous phase of the cream and not strongly adsorbed. These rinsing phases were added to the emulsion aqueous phase. After dialysis and lyophilisation, a dry extract containing the non adsorbed species was obtained. The expected chromatogram of adsorbed species was then obtained by subtracting the chromatogram of the non adsorbing species to the chromatogram of gum Arabic. Figure III-4 displays the comparison of gum Arabic with adsorbed species chromatograms brought back to the same weight fraction.

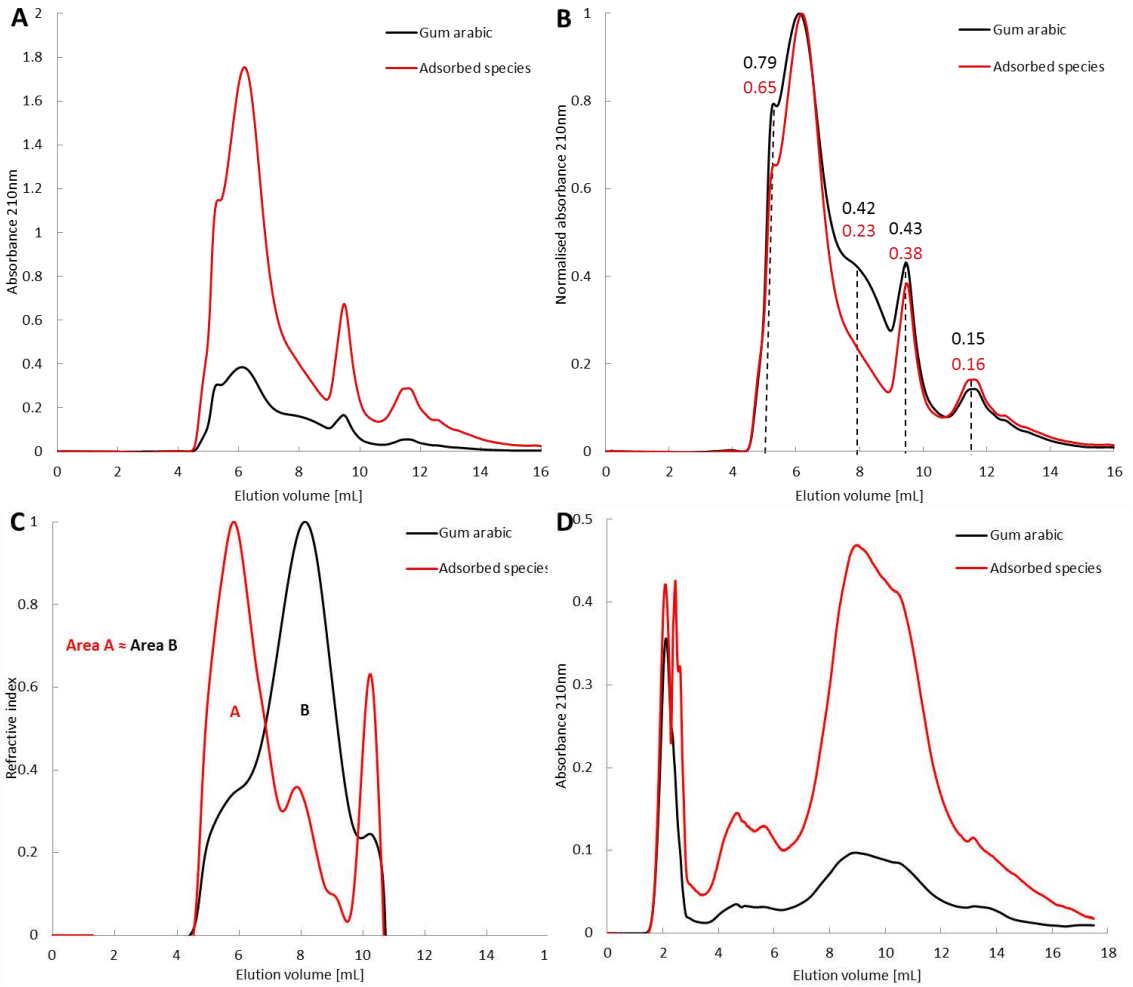

Figure III-4: Comparison of gum Arabic (black line) and gum arabic species adsorbing at oil water interface (red line): (A) size exclusion chromatograms with a UV detection at 210nm, (B): Both chromatograms from (A) are normalized by the highest intensity in order to compare intensity ratios between different peaks, (C) size exclusion chromatograms with a refractive index detection and (D) hydrophobic interaction chromatograms with a UV detection at 210nm.
Adsorbed species chromatograms are obtained by subtraction of the non adsorbed species chromatograms to the total gum Arabic chromatogram and brought back to a concentration of 3w/w% in order to compare the molecular mass composition with that of native gum Arabic solution (same area on refractive index chromatograms figure (C))

Comparison of gum Arabic size exclusion chromatogram with species from the gum adsorbing at the interface chromatogram from Figure III-4A shows that the chromatogram of adsorbed species is much more intense than the chromatogram of gum Arabic with the 210 nm UV detection. This trend is also observed at 280nm (see Figure III-12 in supporting information). This contrasts with the refractive index signal which gives the same area for both samples, which is expected since both solutions have the same concentration in gum species. The contrast in absorbance between adsorbed molecules and gum Arabic shows that for a given elution volume several species with different molar extinction coefficient ε are present. This is consistent with our results from two-

dimensional chromatographic separation of gum Arabic (from another study) with size exclusion as the first separation dimension and hydrophobic interaction as the second separation dimension. Indeed, species collected from a given elution volume in the size exclusion separation yielded a continuum of elution volumes in hydrophobic interaction separation. Interestingly, the sum of all the peaks area ratio of the two chromatograms using a UV detection at 210 nm displayed in Figure III-4A equals to 4.1, which is similar to the protein rate ratio of 3.9 between the two samples. This is consistent with the sensitivity of the 210 nm UV detection to proteins via the peptide bond. We can thus conclude that the interface is largely enriched in the protein rich moieties. In order to assess the size variations of adsorbed species compared to the bulk solution, we normalized both chromatograms to ease peak comparison, as displayed in Figure III-4B. Strikingly, the overall features are similar, which means that the interface remains populated by species of diverse sizes, a result in agreement with the study of Padala and co-workers but contradicts the well-spread idea that only the largest species go to the interface [3], [13]. However, large differences between the native solution and the interface are visible on the refractive index signal, as shown in Figure III-4C. The main component of gum Arabic, which corresponds to intermediate elution volumes and thus molar masses (first population of AGp), is significantly depleted at the interface. On the contrary, the interface is enriched in species corresponding to high molecular masses (AGP: >1000-600kg.mol^{-1}, peak eluted between 4.5 and 7mL) and small molecular mass (second population of AGp and glycoproteins: 250-45 kg.mol^{-1}, peak eluted around 9.5 and 11.5mL). As mentioned in the method section the refractive index detection could not be read after an elution volume of 10.5mL. In figure 4C the mass percentage corresponding to the peak at 11.5mL on the UV chromatogram was not detected. Nevertheless, its adsorbance at 210nm (and 280nm) is not negligible and is amplified in the case of the adsorbed species. The preparative separation of gum Arabic has confirmed that this peak represents a very low weight percentage with a non negligible UV absorption. The fact that its intensity increases in the case of the adsorbed species thus indicates a very high protein rate (above 50w/w%).

According to the molecular weight universal calibration and the concentration repartition from the refractive index profile (assuming that approximately 1 to 2wt% of glycoprotein adsorbed), we estimated that in our emulsion conditions the oil droplets surface were populated of approximately 19-32% in number of glycoproteins, 43-37% in number of the second population of AGp (250-45 kg.mol^{-1}), 16-12% of the first population of AGp (600-250 kg.mol^{-1}) and 22-18% of AGP conjugates (>1000-600kg.mol^{-1}). This estimate shows that gum Arabic stabilized droplets are not primarily covered by large protein-polysaccharides conjugates moieties as discussed in previous studies [5] but by a larger proportion of the smaller molecular weight macromolecules from the gum.

Comparison of gum Arabic and recovered species from the interface hydrophobic interaction chromatograms exhibit important differences as shown in Figure III-4D. The first important observation from this comparison is that the species recovered from the interface present a chromatogram more intense than the gum Arabic at the same concentration. This observation is in accordance with the trend observed in size exclusion chromatography and can be notably assigned to the more important protein rate of this fraction compared to native gum Arabic. The second observation is that the intensity of the first peak, corresponding to the less hydrophobic species which are not retained by the column, is less intense in the case of the species adsorbing at the interface but not negligible. Since the emulsion cream was rinsed, this shows that even weakly hydrophobic species may adsorb at the interface. The third observation is that the interface seems to be composed of a broad range of hydrophobicity, which corresponds to a broad range of elution volumes on the hydrophobic interaction chromatogram.

To summarize, we showed that the interfacial adsorbed film is heterogeneous in molecular mass and hydrophobicity. The high and small molecular masses appear to preferentially adsorb at the interface and these species present a broad range of hydrophobicity. It can be concluded that the interfacial composition largely differs from the bulk solution but is far more complex than what is routinely assumed, for instance a layer of large arabinogalactan-protein conjugates (AGP).

Can we define a mean area per adsorbed species in this adsorption process?

The chromatographic analysis has shown that in the emulsification conditions investigated, amphiphilic species are in large excess. Chromatograms of non adsorbed species (not shown here) show that classes of macromolecules preferentially adsorbing are far from being fully consumed. In a classical adsorption process, for instance involving a surfactant solution, it is expected that a maximal surface density, which corresponds to a mean area per stabilizer, is reached. We decided to measure this quantity in order to check for the behavior of gum Arabic adsorbing species species as a function of several physico-chemical parameters used in an emulsion formulation (pH, gum concentration or ionic strength). In practice these parameters are coupled to one another, since gum Arabic polysaccharides are polyelectrolytes, due to glucuronic moieties. Therefore, varying the gum concentration will also result in changing both pH and ionic strength. Simple comparisons thus require setting two parameters approximatively constant.

Surface densities were calculated from the know amount of gum adsorbed at the droplet surfaces (from the interfacial separation procedure) and the volume-surface diameter of the emulsion (sauter diameter) are presented in Table III-1. Interestingly, there are of the same order of magnitudes as values from previous studies in which emulsions were not rinsed (Padala et al., 2009). Padala et al. argued that such large values of the surface densities compared to small proteins could be explained either by conformational changes or multilayers. Our observations demonstrate that the multilayer hypothesis can not hold, since multilayers can not withstand the successive rinsing steps we imposed on emulsions.

	C_{GA} [wt%]	pH	C_{NaCl} [mol.L^{-1}]	Ionic strengh [mol.L^{-1}]	Protein rate [wt%]	$M_{adsorbed}$ [mg]	d_{32} [µm]	Γ [mg.m^{-2}]
A	5	3.5	0	4.13×10^{-2}	14.2	180±15.1	12.8±0.23	**9.6±0.82**
B	1.6	3.5	0	1.44×10^{-2}	12.6	128±13.1	12.3±0.26	**6.6±0.69**
C	1.6	5	2×10^{-2}	3.36×10^{-2}	11.1	145±13.6	11.8±0.22	**7.1±0.67**
D	1.6	5	0	1.36×10^{-2}	10.7	110±10.1	12.7±0.22	**5.8±0.54**

Table III-1: Total mass of gum adsorbed and surface concentration as a function of pH and ionic strengh.

From Table III-1, we can observe that the surface density depends on both the amount of gum, pH and ionic strength. Comparing emulsions C and D yields the effect of ionic strength while comparing emulsions B and D yields the effect of pH. We can conclude that the value of surface concentration is driven by the magnitude of ionic repulsions. Surface density thus increases when the ionic strength increases and the pH decreases, which turns to decrease ionic repulsions. From emulsion B to emulsion A, the important increase in surface concentration corresponds to a simultaneous increase of ionic strength and decrease of pH, both induced by the increase of gum concentration. These results are in accordance with interfacial tension measurements where a diminution of the pH and an increase in the salinity lead to a decrease of the interfacial tension (see Figure III-16 in supporting information).

Three scenarii can be proposed to interpret these surface density variations: (i) The emulsification conditions correspond to a defect of species with interfacial properties rather than an excess of them. In addition to the chromatographic analysis, which does not support this hypothesis (class of molecules adsorbing far from being fully consumed), comparing emulsions B, C and D shows that even with the same gum concentration, surface concentrations are different. Additionally, it was observed from another study that no limited coalescence mechanisms were observed when using gum Arabic to stabilize emulsions. Indeed, there was no linear evolution between the volume surface diameter of the emulsion droplets and the gum concentration, indicating that we are not in defect of

adsorbing species. (ii) There are differences in film composition induced by the different conditions, leading to packing changes at the interface since species of various sizes are adsorbed (iii) There are conformational changes with the different conditions, leading to changes in surface density resulting from variation of the mean area per adsorbed molecule, similarly to what is observed with adsorbed polymer brushes [24], [25].

Film composition as a function of physico-chemical parameters.

In order to test the second hypothesis (corresponding to surface concentration changes induced by composition changes) a chromatographic analysis of the adsorbed species for the different conditions of Table III-1 was achieved.

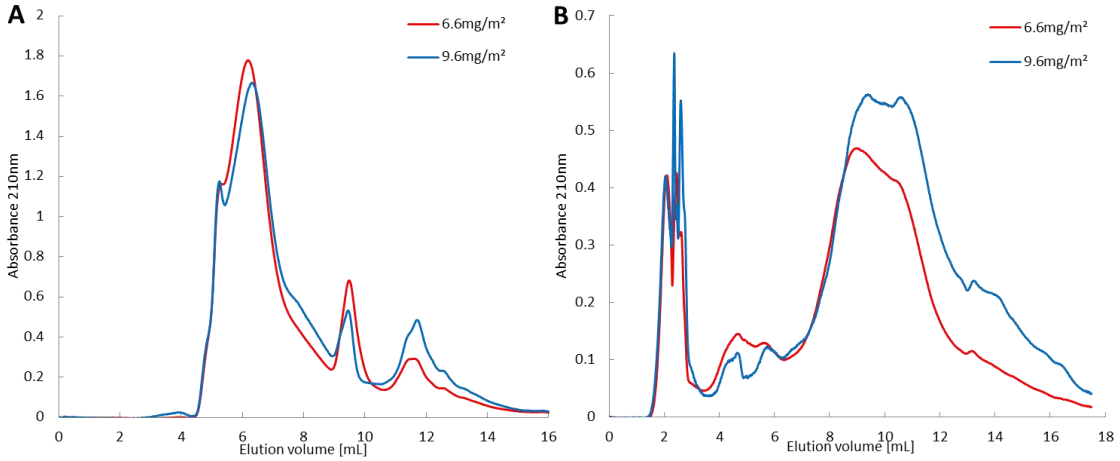

Figure III-5: (A) Sixe exclusion chromatograms and (B) Hydrophobic interaction chromatograms of adsorbed species as a function of the amount adsorbed at oil/water interfaces with a UV detection at 210nm. Chromatograms were obtain by subtraction of the non adsorbed species chromatograms to the total gum Arabic chromatogram and brought back to a concentration of 3w/w% in order to compare the molecular mass composition for each adsorbed film.

First the effect of increasing gum Arabic bulk concentration on the interfacial composition is assessed. As observed from Figure III-5A, the molecular weight distributions for both films composition are similar in shape and sum of all the peak areas under the chromatograms (5.2 for 9.6mg.m^{-2} and 4.85 for 6.6mg.m^{-2}). The molecular mass distribution is slightly more heterogeneous when adsorbing more material at the interface. The area correlates with the protein content (14.2w/w% for 9.6mg/m² and 12.6w/w% of protein for 6.6mg/m²). This small difference in protein rate is consistent with the observation that more amphiphilic species are adsorbed upon increasing the surface density. Similar observations were made from refractive index detection (data not shown here). The hydrophobic interaction chromatograms support consistently that more hydrophobic species are present upon increasing the surface density.

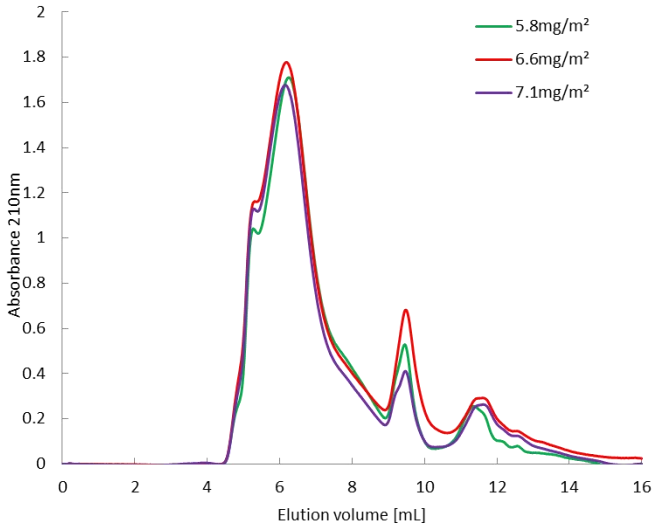

Figure III-6: Sixe exclusion chromatograms of adsorbed species as a function of the amount adsorbed at oil/water interfaces. Chromatograms were obtained by subtraction of the non adsorbed species chromatograms to the total gum Arabic chromatogram and brought back to a concentration of 3w/w% in order to compare the molecular mass composition for each adsorbed film.

Then the effect of ionic strength and pH changes at a constant gum bulk concentration is examined. As observed from the chromatograms of Figure III-6 the composition of the interface appears to only slightly depend on the physico-chemical parameters employed to form an emulsion. Total areas under the curve were also found to be proportional to the protein rate measured for each fraction recovered from the interface. UV detection at 280nm is also in good correlation with the measured protein rate (see Figure III-14 in supporting information). Interestingly, the protein rate does not correlate with the surface concentration, suggesting that the composition varies between different surface densities. However, these composition changes seem overall too small to provide an explanation for the variation of the surface concentration change (hypothesis (ii)).

From the refractive index signal (molar mass repartition as weight percentage) and the total mass adsorbed at the oil/water interface a mean surface area per by adsorbed macromolecules can be estimated. For the more concentrated emulsion (9.6 $mg.m^{-2}$) this area is of 54nm² while it is of 76nm² for the less concentrated one (6.9 $mg.m^{-2}$). This further support the validity of the hypothesis (iii) with gum macromolecules adsorbed at the interface adopting conformational changes.

Film composition changes with reducing the interfacial area.

Emulsion B was centrifuged at 10000g prior to recovering the species from the interface and the aqueous phase of the emulsion. It was observed that the sauter diameter of the emulsion increases after one-minute under centrifugation. The surface coverage is also increased as indicated by the total amount recovered at the interface. However, the total mass of gum recovered at the interface

is less important after centrifugation than without, unveiling a partial desorption of adsorbed gum species as the interfacial area decreases through coalescence events.

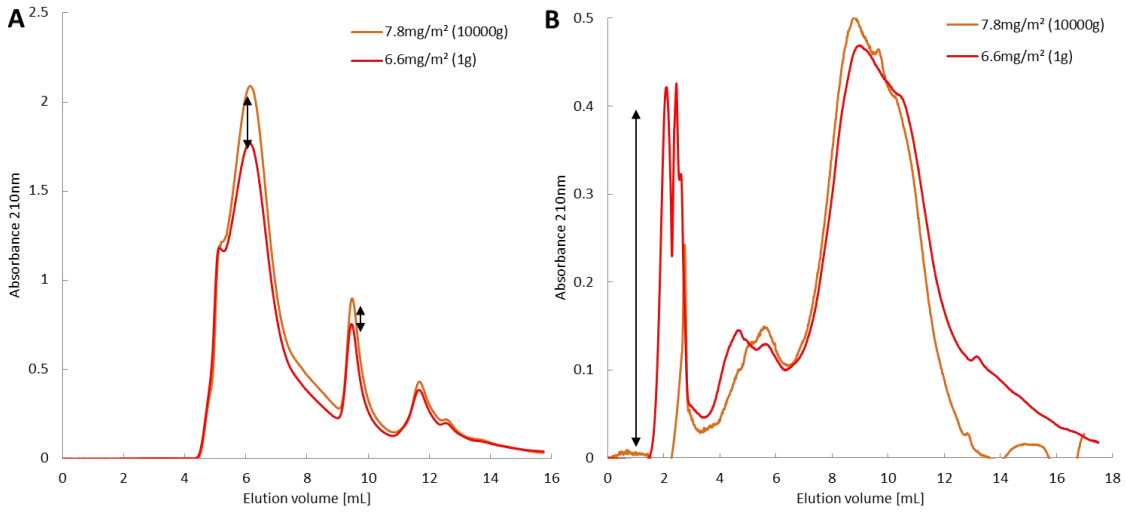

Figure III-7: (A) Sixe exclusion chromatograms and (B) Hydrophobic interaction chromatograms of adsorbed species as a function of the centrifugation speed endure by the emulsion with a UV detection at 210nm. Chromatograms were obtain by subtraction of the non adsorbed species chromatograms to the total gum Arabic chromatogram and brought back to a concentration of 3w/w% in order to compare the molecular mass composition for each adsorbed film.

Comparison of the size exclusion chromatograms in Figure III-7A shows that the interface appears to be enriched with the high molecular weight macromolecules and some of the smaller ones and therefore further depleted in the intermediary molecular masses (Arabinogalactan-peptide). Figure III-7B exhibits a major difference between hydrophobic interaction chromatograms of both samples. Indeed, the first eluted peak completely disappeared in the case of the emulsion that was centrifuged and submitted to coalescence events. A forced reduction of the interfacial area, here achieved by centrifugation and subsequent coalescence, can cause the desorption of the less hydrophilic species, a result consistent with the observation of a much larger protein rate after centrifugation (17.1w/w%) than before (12.6w/w%). Interestingly, the emulsion size distribution was modified only during the first centrifugation step, whilst further centrifugation did not lead up to further evolution. This observation, suggests that desorption of hydrophilic species result from conditions where are forced to coalesce to a given extent, above which emulsions reach a high metastability. It was observed that the adsorption of gum Arabic species is nearly insensitive to surface concentration variations after emulsification whereas the composition might evolve when emulsion droplets coalesce.

Conclusion.

Gum Arabic solutions contain a diversity of polysaccharide/protein species of various sizes and hydrophobicities. When placed in contact with an oil, species from this solution adsorb to the interface. In this work, it is shown that the composition of the interface largely differs from that of the bulk composition of the solution. The interface is enriched in nitrogen, which corresponds to the adsorption of the most proteinaceous species. The interface is also enriched in the minority fractions of gum Arabic corresponding to the large and small sizes but depleted in the predominant moiety corresponding to the intermediate sizes.

Still, the interface remains populated by a large variety of species of contrasted size and hydrophobicity. The adsorption process is activated out of equilibrium, so that most species present at the interface, except the most hydrophilic ones, can be considered as irreversibly adsorbed. This explains the wide use of Arabic gum as a stabilizer for beverage emulsions, which must withstand extensive dilutions.

A significant increase of the interfacial concentration of gum species with a decrease of ionic repulsions is reported, despite a large excess of gum Arabic in the aqueous phase. The ionic repulsions could be varied simultaneously through changes in gum concentration or separately by changing pH or ionic strength. The composition of the interfacial films corresponding to these different interfacial coverages and only relatively subtle variations were observed. Results suggest that these changes in interfacial densities primarily arise from structural changes within the interfacial layer.

Additionally, we suggest that the systematic control of this interfacial density is a key parameter for a rational design of formulations using gum Arabic.

Materials and methods.

Materials.

Powder gum arabic (Acacia senegal) was a gift from Caragum International ® (Marseille, France) and the composition was 2.9% of proteins (Nx6.25), 11% moisture, 3.16% ash and nearly no lipid. Sodium chloride (99.5>% BioXtra), hexadecane (99% ReagentPlus), pentane and hydrochloride acid were purchased from Sigma Aldrich. Distilled water was used for all experiments.

Emulsion ionic strength.

The ionic strength of a solution can be calculated using equation (III-1).

$$I = \frac{1}{2}\sum_i C_i z_i^2 \quad \text{(III-1)}$$

C_i representing the concentration of each ions in solution and z_i representing its valence. In the case of gum Arabic stabilized emulsions all the ions present in equation (III-2) need to be considered. Those include the carboxylic functions present via the glucuronic acid moieties of the polysaccharides, the gum cations, the protons in solution and additional ions during the emulsion formulation. The protein rate of the gum Arabic being around 3w/w%, the contribution of NH_3^+ and COO^- ions of the gum proteins can be neglected. Ionic strength reads:

$$I = \frac{1}{2}([COO^-] + 2^2[Mg^{2+}] + 2^2[Ca^{2+}] + [K^+] + [H^+] + [Na^+] + [Cl^-]) \quad \text{(III-2)}$$

The amount of glucuronic acid (mol) is calculated from the percentage of glucuronic acid (molar mass 194g.mol^{-1}) in gum Arabic taken from the literature (around 14w/w%) [19]. The concentration of deprotonated carboxylic functions is given by equation (III-3) and (III-4).

$$n_{uronic\ acid} = n_{COOH} \quad \text{(III-3)}$$

$$[COO^-] = \frac{n_{COOH} \times \%_{COO^-}}{V_{\Phi_{aqueous}}} \quad \text{(III-4)}$$

Only the deprotonated form of the carboxylic acid moieties will play a role in the ionic strength of the emulsion. The percentage of deprotonated carboxylic acid is dependent on the pKa of glucuronic acid and the pH of the aqueous phase. Glucuronic acid pka is taken equal to 3.2. [26]

$$\frac{[COO^-]}{[COOH]} = 10^{pH-pka} \quad \text{(III-5)}$$

Thus for pH=5 there is approximately 98% of carboxylic acid at deprotonated form while at pH=3.5 the percentage is approximately 67%.

The concentration of gum cations can be calculated from equation (III-6) (illustrated with the concentration of magnesium). The weight percentage of each cation in the gum was determined using ion chromatography ([K]=0.8w/w%, [Mg]=0.16w/w%, [Ca]=0.55w/w%).

$$[Mg^{2+}] = \frac{wt\%_{Mg} \times m_{GA}}{M_{Mg} \times V_{\Phi_{aqueous}}} \quad \text{(III-6)}$$

Concentrations of the sodium and chloride ions are dependent on the amount of sodium chloride (NaCl) and/or hydrochloric acid (HCl) added to the aqueous phase. Finally the concentration of free protons is provided by the pH value.

$$[H^+] = 10^{-pH} \quad \text{(III-7)}$$

Emulsion preparation.

Oil-in-water emulsions stabilized by gum arabic were prepared using a rotor/stator device (Ultra-Turrax ®). Alkane oil (pentane or hexadecane) were used as the organic phase. We showed that the nature of the oil did not influence the composition of the interface (see Figure III-10 in supporting information). Emulsions were formulated as follow: 40v/v% of oil was dispersed in gum Arabic aqueous solution. Gum Arabic solutions were prepared at different concentrations and centrifuged under 19000g to remove insoluble materials. Then the pH or the salt concentration of each solution was adjusted by adding small amounts of HCl at 1mol.L^{-1} or NaCl at 3 mol.L^{-1}. Both phases were then mixed during one minute at 10000rpm.

Interfacial separation.

Gum arabic amphiphilic species were separated through an emulsion mediated separation technique. Emulsions of pentane in water stabilized by Arabic gum were formed following the aforementioned protocol. Emulsions were then placed in a separative funnel, diluted with distilled water saturated with pentane (in order to avoid pentane to diffuse from oil droplets into the aqueous phase) and let to cream. Aqueous phase was recovered and the emulsion cream was rinsed again in order to remove all species that could be trapped between droplets without being strongly adsorbed at an interface. The aqueous phase containing all the species that did not adsorb was then dialyzed against water and lyophilized. The final cream was dispersed in water and freeze-dried.

A mass balance on the recovered species was carried out on each batch in order to calculate the surface concentration of the droplets.

Drop size distribution.

Droplet size distributions of emulsions were measured using a static light scattering instrument (Mastersizer 3000, Malvern ®). The laser wavelength was of 633nm. An emulsion sample was dispersed in recirculated water until at an obscuration rate between 1-10% was obtained to avoid multiple scattering. The average droplet diameter was determined as the volume-surface mean diameter d[3,2). Each measurement was repeated in triplicate at room temperature.

Size exclusion chromatography.

Size exclusion chromatography was used to separate Arabic gum species as a function of their relative hydrodynamic volume. A 7.8mmx300mm BioSuite 450Å SEC column (Waters ®) packed with 8µm porous silica beads was used. The average pore size of the silica beads is 450Å.

The separation was performed on an Alliance HPLC unit (Waters 2695 separations module), a 0.5mol.L^{-1} NaCl aqueous solution at 25°C was used as the eluent phase at a flow rate of 0.8mL.min^{-1}. Each sample was filtrated with a nylon 0.2µm membrane and a volume of 50µL was injected. UV detection was performed at 210 nm and 280nm using a Waters® 2487 UV detector. A refractive index detection was used to measure the mass percentage of each eluted moity (Waters® 410 differential refractometer). The refractive index detection was only available until 10.5mL of elution due to a negative peak appearing from the difference in refractive index between the sodium chloride of the eluent phase and the water from the injected sample. The column was calibrated using branched dextran standards (Waters ®). [27]

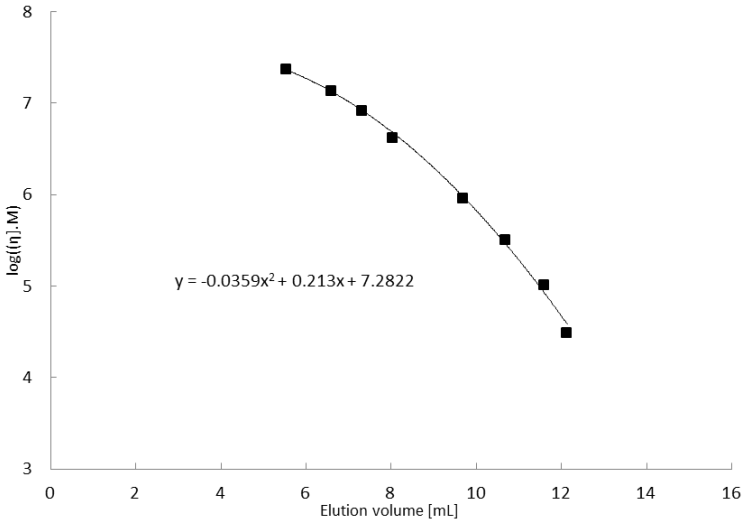

Figure III-8: Universal calibration curve (performed with branched dextran of known molecular weight) for size exclusion separation on Biosuite 450Å SEC column (waters) with a low rate of 0.8mL/min and 0.5M NaCl as the eluent solution.

Variation coefficients for each detection were calculated from three injections of the same sample. For UV detection at 210nm a 0.3% variation coefficient was measured. It was of 0.5% for UV detection at 280nm and of 13% for the refractive index detection.

UV detection at 280nm is more sensitive to the rate of cyclic amino acid present within the gum polypeptidic moieties (tyrosin and phenylalanine). Cyclic amino acids however are not the predominant amino acid moiety in gum Arabic, as already observed in the literature [5], [14], [28]. UV detection at 210 nm is mostly sensitive to $\pi \rightarrow \pi^*$ transitions from C=O bond of the amides

linkages between amino acid moieties, and from those of carboxylic moieties present onto the polysaccharides backbones and of reducing sugar units at the end of polysaccharides chains.

Hydrophobic interaction chromatography.

Hydrophobic interaction chromatography was used to separate Arabic gum fractions as a function of their hydrophobic properties. A 7.5mmx75mm 10µm Biosuite Phenyl column (Waters ®) which consist of phenyl groups bonded to a metacrylic ester based polymeric resin. The average pore size of the column is 1000Å to accommodate macromolecules with high molecular weight. A gradient of salt concentration is required to create a "salting out" effect and progressively desorb the hydrophobic species. The less hydrophobic species are eluted first with highest salt concentration, while the more hydrophobic species are the last one eluted.

The separation was performed on an Alliance HPLC unit (Waters 2695 ® separations module). The mobile phase was composed of a solution of NaCl at a constant flowrate of 0.5mL.min^{-1}. A continuous time gradient of salt concentration between 4mol.L^{-1} and 0 was applied during 22minutes followed by distilled water during 12 more minutes (Figure II-10). Each sample was filtrated with a nylon 0.2µm membrane and a volume of 30µL was injected. UV absorbance was measured at 210 nm and 280nm (Waters® 2487 UV detector). We observed that UV absorbance (especially at 210nm) was very sensitive to NaCl concentration. The baseline was not constant along the separation time. In order to correct this deviation the chromatogram of distilled water injected in same elution conditions was subtracted to each measured chromatogram.

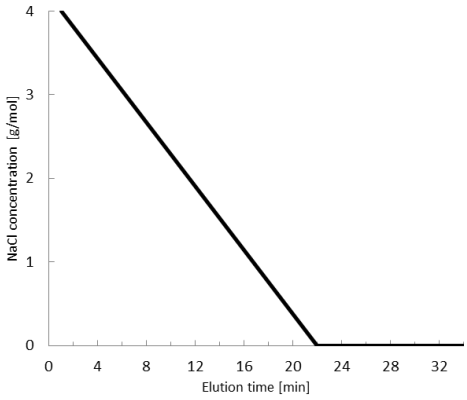

Figure III-9: NaCl elution gradient used for hydrophobic interaction chromatographic separation.

Variation coefficients for each detection were estimated from comparison of three injections of the same sample. A 6% (resp. 11%) variation coefficient was observed at 210nm (resp. 280nm).

Interfacial film composition.

The surface cencentration (in $mg.m^{-2}$) in emulsions was determined using equation (III-8). The amount of recovered mass (m_{ads}) was determined from the mass balance from the interfacial separation procedure.

$$\Gamma = \frac{m_{ads} d_{[3,2]}}{6V_{oil}} \quad \text{(III-8)}$$

Chromatograms of adsorbed species at interfaces were determined using the following procedure: a native gum Arabic sample and the species recovered from the aqueous phase of the emulsion (non adsorbed species) were solubilized in water at a concentration of 3w/w%. The same volume for each solution was injected into both chromatographic columns (SEC and HIC). For both chromatographic techniques, the total area of chromatograms has been found proportional to the species concentration of each sample (see Figure III-11 in supporting information). Knowing from the interfacial separation procedure the mass percentage of macromolecules from the gum that did or did not adsorbed, the chromatogram of the species recovered from the aqueous phase was multiplied by its mass percentage. In order to obtain the chromatogram of the adsorbed species the chromatogram of the non adsorbed species was subtracted to that of the gum Arabic. Finally in order to compare each chromatogram between them, the resulting chromatogram was multiplied by one hundred divided by its mass percentage determined from the interfacial separation procedure (percentage of gum macromolecules that adsorbed from the amount of gum used in the emulsion).

Nitrogen rate analysis:

Nitrogen rate analyses were performed at the Laboratoire de Coordination Chimique (Toulouse, France). A Perkin-Elmer 2400 CHN series II was operated at 1050 °C with oxygen as carrier gas. Each sample was analyzed in duplicate. Protein fraction rate was deduced from the nitrogen rate through the protein conversion number calculated from the gum Arabic proteins amino acid distribution [17].

$$\%_{protein} = \%_{nitrogen} \times 8.14 \quad \text{(III-9)}$$

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Supporting information.

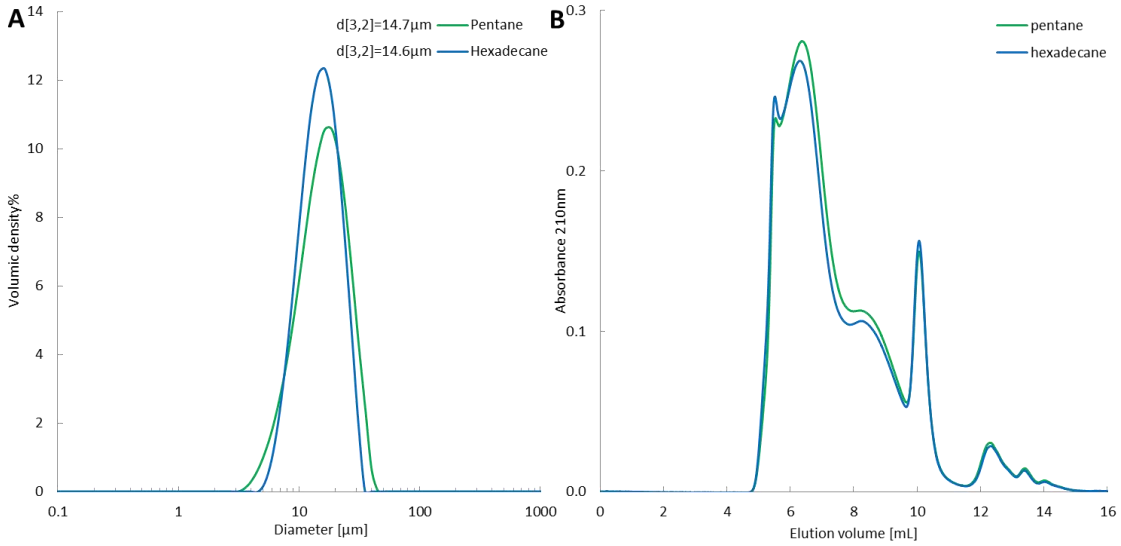

Figure III-10: (A) Emulsion size distribution with either pentane or hexadecane as the oil phase using the same formulation, (B) Same emulsions aqueous phase size exclusion chromatograms.

Small differences in interfacial composition may arise from the fract that although both emulsions exhibit similar Sauter diameters, their size distribution are slightly different, with the emulsion with pentane being more polydisperse. Nevertheless their interfacial composition is nearly the same.

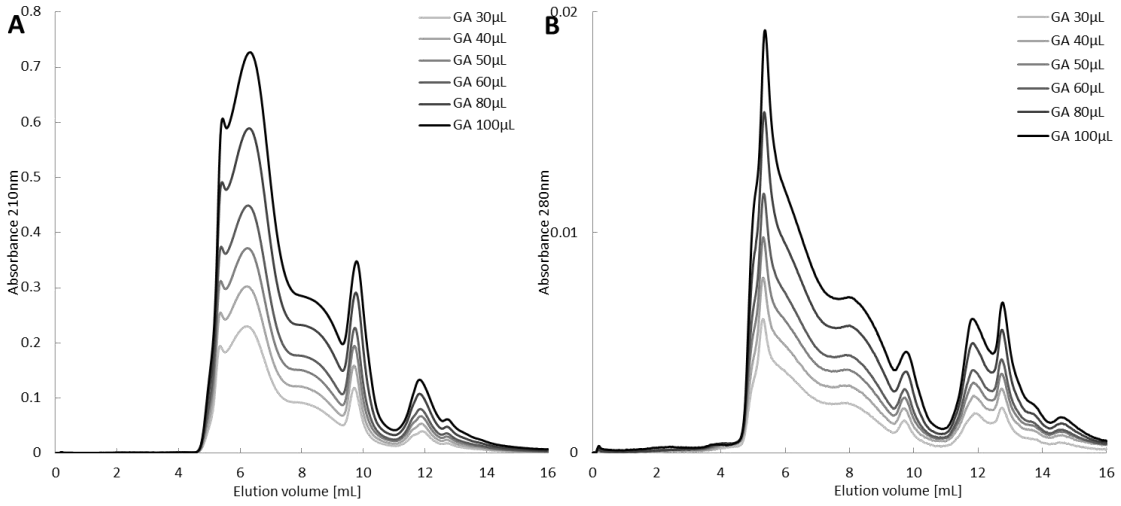

Figure III-11: Size exclusion chromatogram of gum arabic at 3w/w% by varing the injected volume. (A) UV detection at 210nm, (B) UV detection at 280nm.

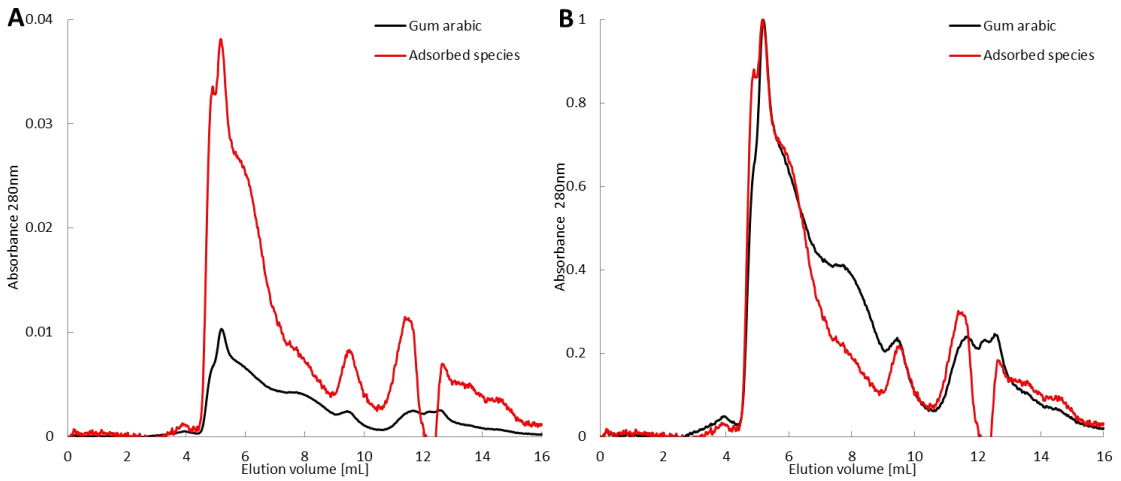

Figure III-12: Comparison of gum Arabic (black line) and gum arabic species adsorbing at oil water interface (red line) size exclusion chromatograms with a UV detection at 280nm. (A): Adsorbed species chromatograms was obtain by substraction of the non adsorbed species chromatograms to the total gum Arabic chromatogram and brought back to a concentration of 3w/w% in order to compare the molecular mass compositon with gum arabic. (B): Both chromatogram from (A) were normalized by the higher intensity in order to compare intensity ratios between different peaks.

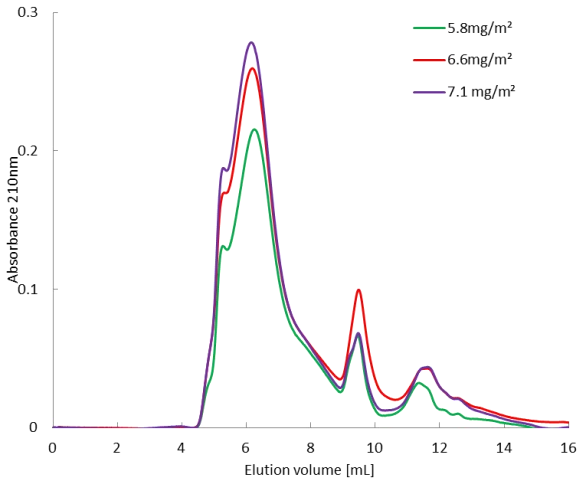

Figure III-13: Sixe exclusion chromatograms of adsorbed species as a function of the amount adsorbed at oil/water interfaces. Chromatograms were obtain by substraction of the non adsorbed species chromatograms to the total gum Arabic chromatogram.

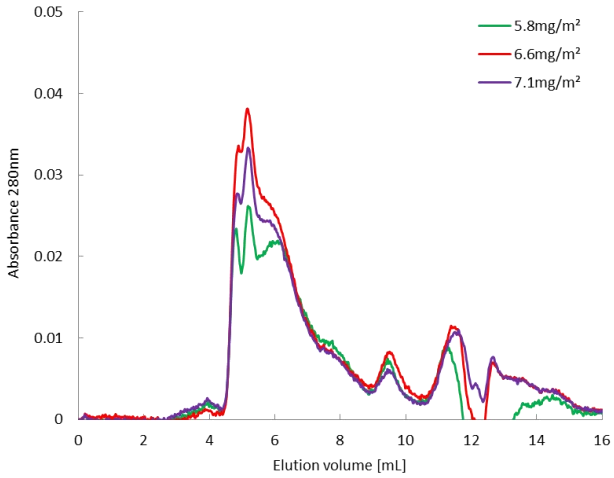

Figure III-14: Sixe exclusion chromatograms of adsorbed species as a function of the amount adsorbed at oil/water interfaces with UV detection at 280nm. Chromatograms were obtained by subtraction of the non adsorbed species chromatograms to the total gum Arabic chromatogram and brought back to a concentration of 3w/w% in order to compare the molecular mass composition for each adsorbed film.

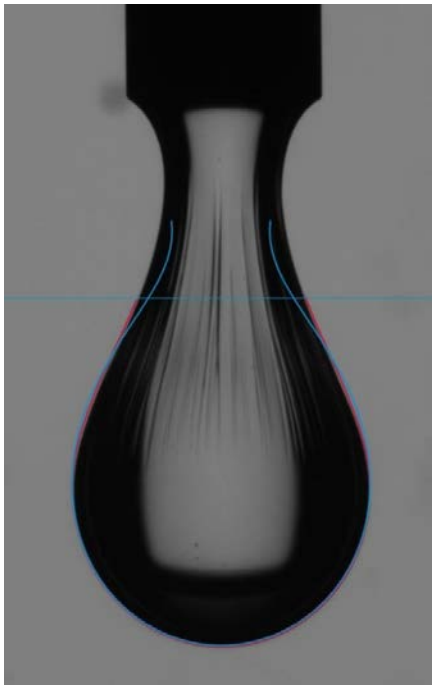

Figure III-15: Picture of a pendant drop covered with gum arabic moities that suffered from a volume reduction

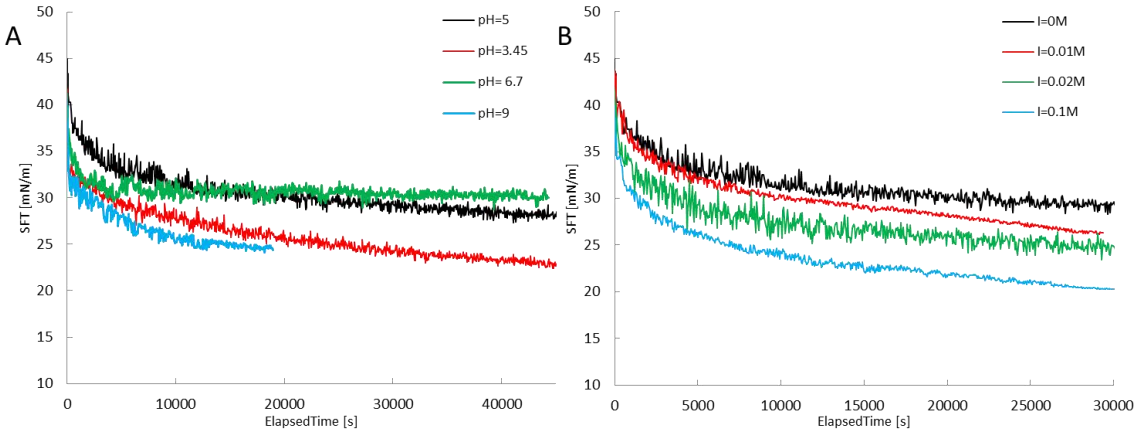

Figure III-16: Evolution of interfacial tension between aqueous solution of gum arabic at 0.5wt% and n-hexadecane as a function of time by varying (A) the pH of the aqueous phase or (B) the salinity of the aqueous phase by addition of NaCl.

According to interfacial tension measurements between gum Arabic solutions and hexadecane, physico-chemical parameters appeard to influence the adsorption of gum Arabic species at the interface. This observation might be either the results of an increase in the amount of gum amphiphilic species adsorbed (isotherms of adsorption), or the difference in the type of macromolecules adsorbed (chromatographic analysis) or a combination of both.

Chapter IV- Interfacial structuration in gum Arabic stabilized emulsions

Abstract.

Gum Arabic, a natural hydrocolloid highly soluble in water, is extensively used to provide metastability to oil/water emulsions. We showed that gum Arabic is composed of a complex mixture of polysaccharides and proteins, which presents a concentration dependent, multiscale structure in solution. The composition of adsorbed gum Arabic interfacial film is enriched in the protein rich gum species and we observed that surfaces densities are highly dependent on physico-chemical parameters employed during emulsification, while only minor composition changes in the adsorbed layer were observed. Gum Arabic adsorbed species can thus adopt conformational changes when adsorbed at oil/water interface. Here, we discuss the surprising metastability of emulsions stabilized by gum Arabic, which can display exceptional resilience to destabilization induced by dilution or ultra-centrifugation but also poor stabilization depending on the formulation used. Species recovered from the interface are partially aggregated when dissolved in water and the magnitude of this aggregation depends on emulsification conditions, notably surface concentration and protein content. Chromatographic analysis on these adsorbed species show aggregates are composed mainly of glycoproteins and small mildly hydrophobic arabinogalactan peptides under moderate aggregation conditions and include the larger arabinogalactan-protein conjugates at higher aggregation conditions. Small angle neutron and X-ray scattering were performed on solutions of species recovered from oil/water interfaces and the aggregate structure was investigated. We show that the conformation at both small and large length scales differ from native species. Using contrast-matched conditions, we investigated the interfacial structure of gum stabilized oil/water emulsions. We found that the structure at large length scale matched the structure in solution at the relevant concentration, which largely varied with formulation conditions. On the contrary, the structure at small length scales is radically different and corresponds to disordering of the native structures.

Introduction.

Gum Arabic is a natural product issued from acacia trees in sub-Saharan countries. This hydrocolloid is extensively used for its interfacial properties (binding and stabilization) in several applications such as the stabilization of oil-in-water emulsions for the food industry. As a result, gum Arabic has been extensively studied in the literature, notably in respect to its composition and structure. Gum Arabic is described as a complex mixture of covalently linked protein and polysaccharides macromolecules with various size, hydrophobicity and protein rate [1]–[3]. Gum composition was notably studied using chromatographic separation techniques such as size exclusion and hydrophobic interaction. Using hydrophobic interaction chromatography the gum was described as composed of three fractions: an arabinogalactan polysaccharide fraction (88%), a protein-polysaccharide conjugates fraction (10%) and a glycoprotein fraction (2%) [1], [2], [4]–[8]. In a previous work we studied the

macromolecular composition and structure of the gum using a two dimensional chromatographic separation combined with small angle scattering experiments (SAXS and SANS). Gum Arabic in solution was described as a multiscale structure with two populations of arabinogalactan-peptide (AGp) differing in sizes and protein content and several glycoproteins with a variable content of hydrophobic amino-acids. Conjugates of these different species were also present and correspond to large arabinogalactan-protein conjugates (AGP).

The use of gum Arabic to stabilize oil/water emulsions was also extensively studied. The protein content of gum Arabic was shown as playing a key part in the stabilizing properties of the gum [9], which lead to the conclusion that polypeptide moieties are responsible for adsorption properties. The high molecular weight conjugate fraction (AGP) was described as responsible for the emulsifying properties of the gum [9], with the carbohydrate moieties providing strong steric repulsions and the polypeptide chain adsorbing at interfaces. Nevertheless, emulsifications using recovered gum Arabic fractions from size exclusion chromatography (size) or hydrophobic interaction (protein content) showed that using a combination of both small and the high molecular weight species provided the best emulsion metastability [10]. This important observation suggests that the origin of gum Arabic emulsifying properties arise from a combination of macromolecules and not just the high molecular weight conjugates. Padala and co-workers observed, from a chromatographic analysis of an emulsion aqueous phase after emulsification, that species from all the size distribution of the gum could adsorbed in various proportions [11]. In order to fully elucidate the composition of gum Arabic adsorbed film at oil/water interface in emulsions we performed a study based on chromatographic analysis. We showed that the interface is enriched in protein rich species that display a broad distribution of sizes, which corresponds to both glycoproteins and AGP conjugates. More importantly, we showed that the surface concentration, contrarily to the interfacial composition, largely depends formulation conditions and notably increased with increasing gum concentration of decreasing ionic repulsions through increasing ionic strength or decreasing pH.

Interfacial measurements on gum Arabic were also performed by many research groups. It was shown that the lowering of interfacial tension by gum Arabic was slow due to the high molecular weight of the gum species, which need time to diffuse to the interface, adsorb and rearrange [12], [13]. Moreover some authors mentioned and studied the interfacial rheology behavior of adsorbed gums species. Dickinson et al were the first one to mention that adsorbed gum Arabic films exhibits high surface shear viscosity [14]. The rheological behavior of gum adsorbed species was found to present high viscosity as compared to other systems [15]. Overall, many studies have reported the ability of gum Arabic to form cohesive interfacial films, notably upon drying (Sanchez et al. 2017).

So far gum Arabic was described as a mixture of different species adsorbing at oil/water interface, providing steric repulsion, with the interfacial film exhibiting an elastic behavior. However, there is a lack of direct studies of the interfacial layer structure in gum Arabic stabilized emulsions.

We thus decided to further study the stabilization mechanisms at play when using gum Arabic to stabilize oil-in-water emulsions and notably the interfacial structure. First, we discuss the surprising metastability of gum stabilized emulsions. Then we discuss the surprising behavior in solution of adsorbed species, which were recovered from the interface and characterized through a dual chromatography analysis. Finally, small angle neutron scattering experiments are presented with the aim to study the structure of recovered species from the interface and adsorbed gum films.

Results and discussion.

The surprising metastability of gum Arabic stabilized emulsions

Figure IV-1: Gum Arabic-stabilised (20v/v%) oil-in-water emulsion with millimeter droplets, which remained metastable for 3 years.

Oil-in-water emulsions may display outstanding metastability when gum Arabic is dissolved in water. The stabilizing properties of gum Arabic can be vividly observed through two simple experiments. First, a hexadecane-in-water emulsion composed of a 0.6w/w% solution of gum Arabic in water at pH 3.5 was formed, using a stir-bar rotating at 1000 rpm for 10 min. A relatively narrow size distribution of millimeter droplets was observed after visible coalescence events. Such an emulsion has kept a perfect metastability over the past three years, although submitted to several dilutions, temperature changes and bacteria growth in the vial (Figure IV-1). Such a remarkable metastability of coarse emulsions has already been observed with some particle-stabilized interfaces (pickering emulsions), where particles are irreversibly adsorbed [16], [17].

Second experiment consists of ultracentrifugation tests on gum Arabic-stabilized micron-size emulsions (6µm, pH=3.5 and gum concentration of 2 and 4 w/w%). No discernable change could be observed after several minutes test runs at accelerations larger than 250,000 *g*. In both experiments,

emulsion metastability was easily preserved despite drastic mechanical and physico-chemical perturbations.

However, it is also strikingly easy to fail at producing a metastable emulsion when using gum Arabic as the stabilizer. For instance, working with a gum concentration under 1w/w% for a volume fraction of dispersed phase (oil) of 20v% and without modifications of the physico-chemical parameters of the gum solution (pH or salt concentration) resulted in the rapid formation of an oil layer on top of a coarsening emulsion. Such a large difference in outcome underlines the key role of formulation and calls for further investigations.

We have shown in another study that gum Arabic contains amphiphilic species that adsorb irreversibly at oil/water interfaces. In good formulation conditions, this suggests a similar behavior between gum stabilized-emulsions and particle-stabilized emulsion, which is further supported by the visual aspect of millimeter-size emulsions such as that illustrated in Figure IV-1. A key feature of particle-stabilized emulsions is the limited-coalescence regime. This regime is observed in the case of irreversibly adsorbed stabilizers and with a default of stabilizer compared to the total available surface area created during emulsification. Emulsion droplets will coalesce until the interface is fully covered by the stabilizer. This mechanism typically takes place within minutes in particle-stabilized emulsions. Quantitatively, the limited coalescence regime corresponds to a linear variation of the inverse of the droplet Sauter diameter with the amount of stabilizer and a decrease of the emulsion polydispersity as coalescence proceeds ($d[3,2] \propto 1/C$). We have prepared several emulsions containing increasing amounts of gum Arabic using a dispax rotor/stator at 15000rpm during 3 minutes. The water pH was adjusted at pH=3.5 by adding HCl. The droplet size distribution was measured using static light scattering with a Malvern Mastersizer. The droplet Sauter diameter measured a few minutes after emulsification, was found to be independent of gum Arabic concentration. This diameter hence corresponds to the minimal diameter achievable under the above emulsification conditions. In contrast to what has been observed in a limited coalescence regime, emulsion coalescence proceeds over weeks rather than minutes, suggesting that there always exists a significant barrier hindering droplets coalescence. We have actually observed that the droplets' diameter remained independent of gum Arabic concentration over time for a gum concentration larger than a threshold value of 2%. In particle-stabilized emulsions, this regime would correspond to the stabilizer-rich regime, for which the surface area generated is immediately covered by the stabilizer. We would therefore expect to observe a limited coalescence regime at lower gum concentrations. Instead, we observed emulsion coarsening over weeks, with a progressive broadening of the emulsions size distributions until the measurement could not be performed due to an abundant oil layer on top of the sample. Therefore, a finite mean droplet size is never reached for emulsions prepared at low gum Arabic concentrations. It can be concluded that gum Arabic

emulsions don't exhibit a limited coalescence regime, despite irreversible adsorption of the stabilizing species. However, these experiments demonstrate again that gum Arabic may behave either as a high efficiency stabilizer or as a poor one. Since a very good metastability is reached at the larger gum Arabic concentrations, a first interpretation of this apparent metastability could be provided by the viscoelastic properties of the bulk that would inhibit film drainage between colliding droplets (due to the existence of a yield stress) and subsequently coalescence events. However, it is observed that emulsion metastability is preserved and even reinforced upon dilution, discarding such a mechanism.

We then consider and alternate hypothesis stemming from a set of data related to the interfacial coverage of gum arabic stabilized emulsions. It was demonstrated, through a chromatographic analysis, that despite an excess of stabilizing species in solution, the interfacial coverage of oil droplets drastically varied with gum concentration, while the interfacial composition was nearly unchanged. We observed a similar trend when increasing ionic strength or decreasing pH, i.e. when decreasing the magnitude of ionic repulsions. This result highlights the predominant role of the interfacial structure on emulsion metastability, leading us to relate this structure to the coverage density.

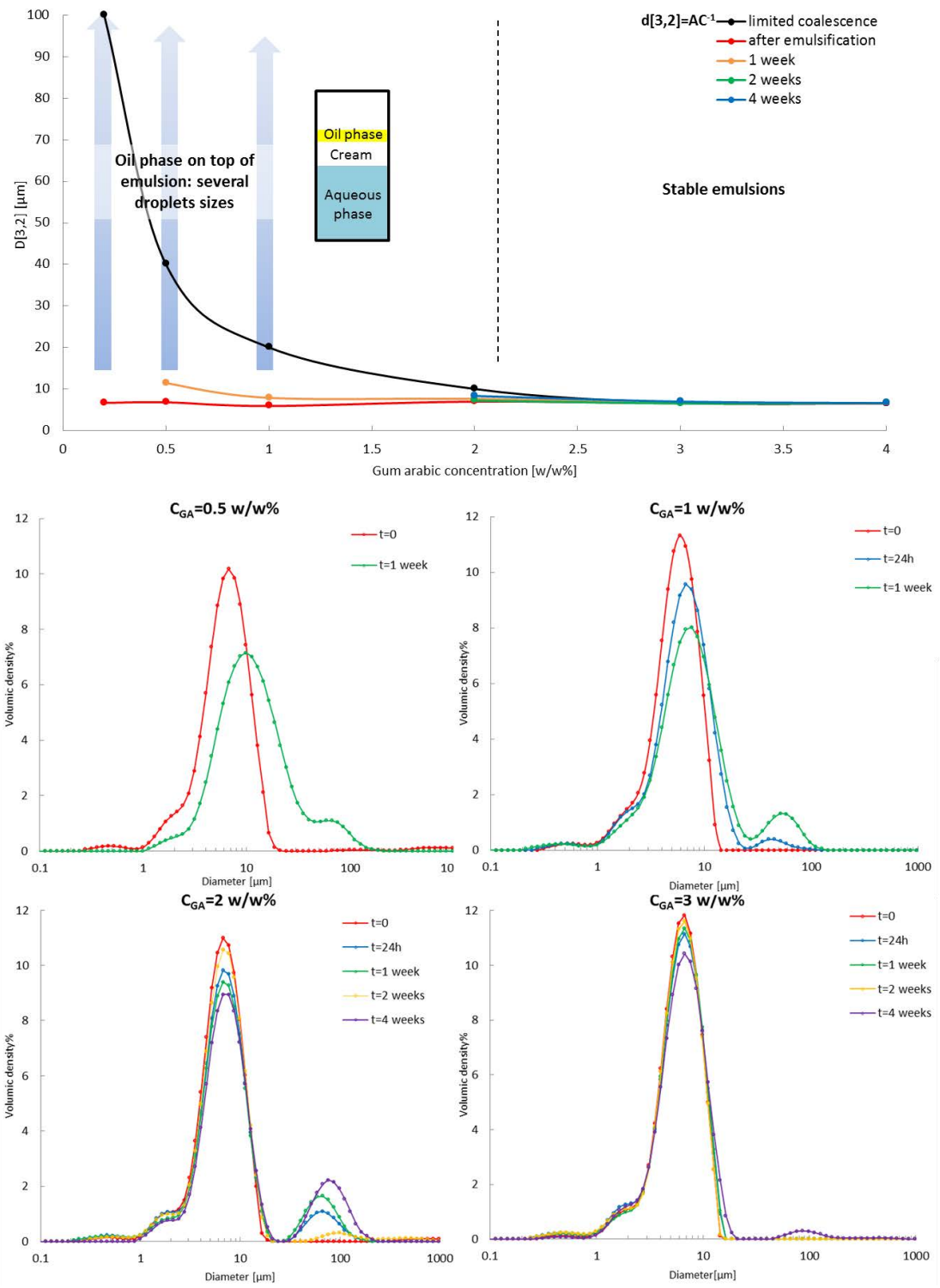

Figure IV-2: Evolution of gum arabic stabilized emulsions (oil volume fraction ϕ=20% (hexadecane), pH=3.5) d[3,2] and size distributions over time compared with limited coalescence theoretical curve (black line). Emulsions were mixed using a rotor/stator dispersion unit (Dispax magic lab IKA ®). Prefactor A is arbitrarily set.

Recovery of gum Arabic adsorbed species from interfaces

Gum Arabic is a complex mixture of polysaccharides/protein species displaying a broad range of sizes and hydrophobicity [1]–[3]. As a consequence, the interface is also of complex composition which has been investigated with the help of a double chromatographic analysis technique. A method has been developed to recover gum species adsorbed at the droplet interfaces, which govern emulsion metastability. This emulsion mediated separation method consists in preparing a pentane-in-water emulsion using gum Arabic as a stabilizer. Emulsions were either left to cream in a separating funnel or centrifuged. The resulting concentrated emulsion was rinsed with distilled water three times. Finally, the whole emulsion was freeze-dried, removing both pentane and water. Adsorbed species were collected as a powder in a vial after freeze-drying. Surprisingly, dissolving this powder in water yielded a cloudy solution, in contrast with the fully transparent gum Arabic solution. Since the cloudiness drastically varied with formulation changes for a given oil content, we have excluded the hypothesis of oil contaminants as its origin. It must thus originate from colloidal aggregates that are not present in the original gum solutions but form at the oil/water interface. These aggregates are a signature of structural changes at the oil/water interface.

The cloudiness of the solutions was found to strongly depend on the formulation and separation conditions, ranging from nearly native gum-like transparent solutions to milky solutions. The transmittance of the solutions has been measured using a spectrophotometer, at two gum concentrations and different separation conditions, under gravity in a separative funnel or under centrifugation. Figure IV-3 shows that the solution transmittance at 800nm varies with both parameters (see Figure IV-9 SI for the 200-800 nm measurements) as well as the Sauter diameter of the initial emulsion droplets. It is important to notice that centrifugation also resulted in an increase of the droplet diameter (see Figure IV-10 SI), indicating a decrease of the overall interfacial area. Furthermore,it was previously shown that this area decrease forces some weakly amphiphilic species to desorb from the interface, leading to an increase of protein content at the interface. This correlation between the cloudiness of the adsorbed species solution and protein content suggests that the solution contain aggregates originating from hydrophobic aggregation at the interface between protein rich species. The literature extensively mentions the film-forming properties of gum Arabic. Erni et al. performed measurements of interfacial rheology of gum Arabic adsorbed films [15]. They have observed that interfacial films possess an elastic behavior unlike adsorbed modified starch.

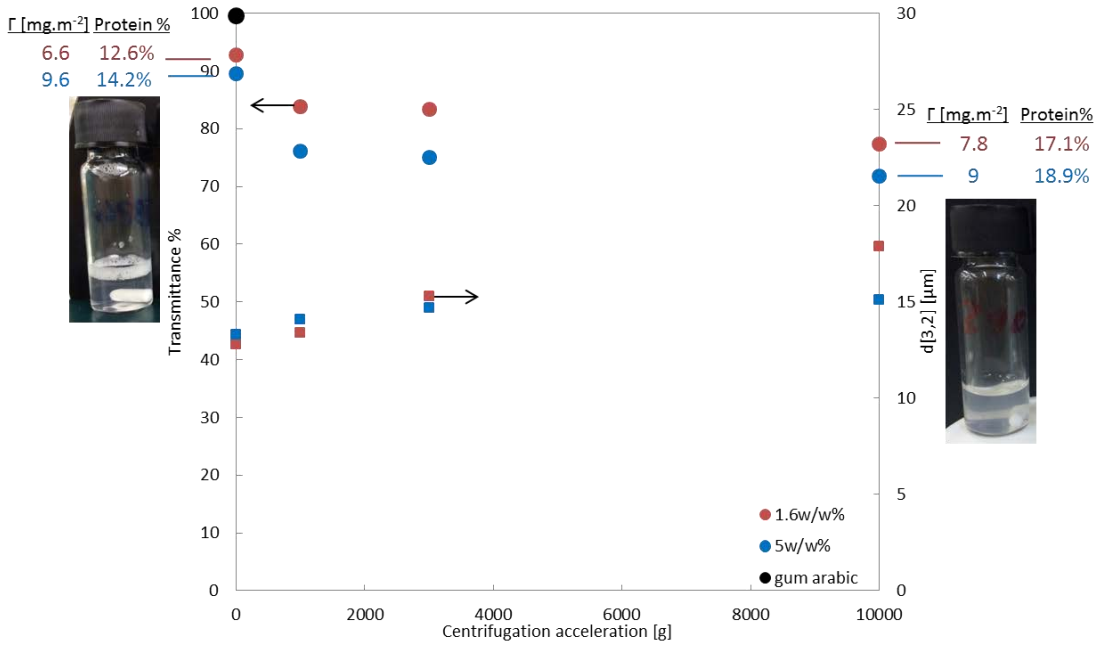

Figure IV-3: Evolution of recovered species from the interface solution (1w/w%) transmittances (circles), and of volume-surface mean diameter of emulsions after rinsing steps (squares) as a function of gum concentration in the emulsion (1.6 or 5w/w%) and speed of acceleration during rinsing steps (1g corresponds to the case without centrifugation and rinsing in a separating funnel). Transmittance of a native gum Arabic solution at 1w/w% is reported in the figure (100%). Protein rate (w/w%), and surface density Γ(mg.m^{-2}) for recovered fraction rinsed at 1g and 10^3 g was measured and is indicated in the figure.

It can be seen from Figure IV-3 that the droplet size evolution is more significant in the case of gum Arabic stabilized emulsion with a concentration or 1.6w/w% than with 5w/w%. Emulsions stabilized by 5w/w% of gum better resisted to coalescence when submitted to centrifugation. As also seen from Figure IV-3 the protein rate as well as the surface concentration are higher at larger gum concentration (5w/w%) in the case where no centrifugation was applied. Both coverage densities and protein content seem to correlate with the formation of aggregates at the oil/water interface.

What are the aggregates made of?

Filtrating these cloudy solutions of solubilized adsorbed species with 0.2µm membranes yielded transparent solutions similar to gum Arabic solutions. The aggregates are therefore larger than 200 nm. In order to identify the composition of these aggregates, a dual chromatographic analysis of both the non-adsorbed and adsorbed species has been performed, using size exclusion (Figure IV-4) and hydrophobic interaction chromatography (Figure IV-11 in Supporting Information). Chromatograms of adsorbed species have been derived subtracting the chromatogram of non-adsorbed species recovered from the aqueous phase from that of the native gum chromatogram.

These calculated chromatograms were compared to the chromatograms of recovered adsorbed species that were re-dissolved in water (yielding cloudy solutions) and filtrated at 200nm. Two parameters are investigated here, the native gum concentration in solution (1.6 and 5 w/w%) and the centrifugal acceleration (1 and 10^4 g) applied during phase separation (leading to 4 pairs of chromatograms for each chromatography technique). As a general trend, the area of the chromatograms of recovered species (dotted line on the graphs) is of lower intensity than the calculated chromatograms (continuous line). This difference corresponds to the mass loss resulting from the filtration of the aggregates formed at the interface. This method therefore provides a relevant tool to unravel the composition of the aggregates forming at the oil water interface.

In size exclusion chromatography the molecules with the higher molecular weight are eluted first. In order to quantify the molecular weight of gum Arabic species, a universal calibration was performed using branched dextran standards. The scale of molecular weight of gum species related to their elution volume appears on top of the chromatograms in Figure IV-4. Chromatograms of Figures 4A and 4B correspond to the case with no centrifugation (gravity driven separation), whereas chromatograms of Figure 4C and 4D correspond to the case where the two liquid phases were centrifuged at 10^4 g, hence submitted to coalescence events.

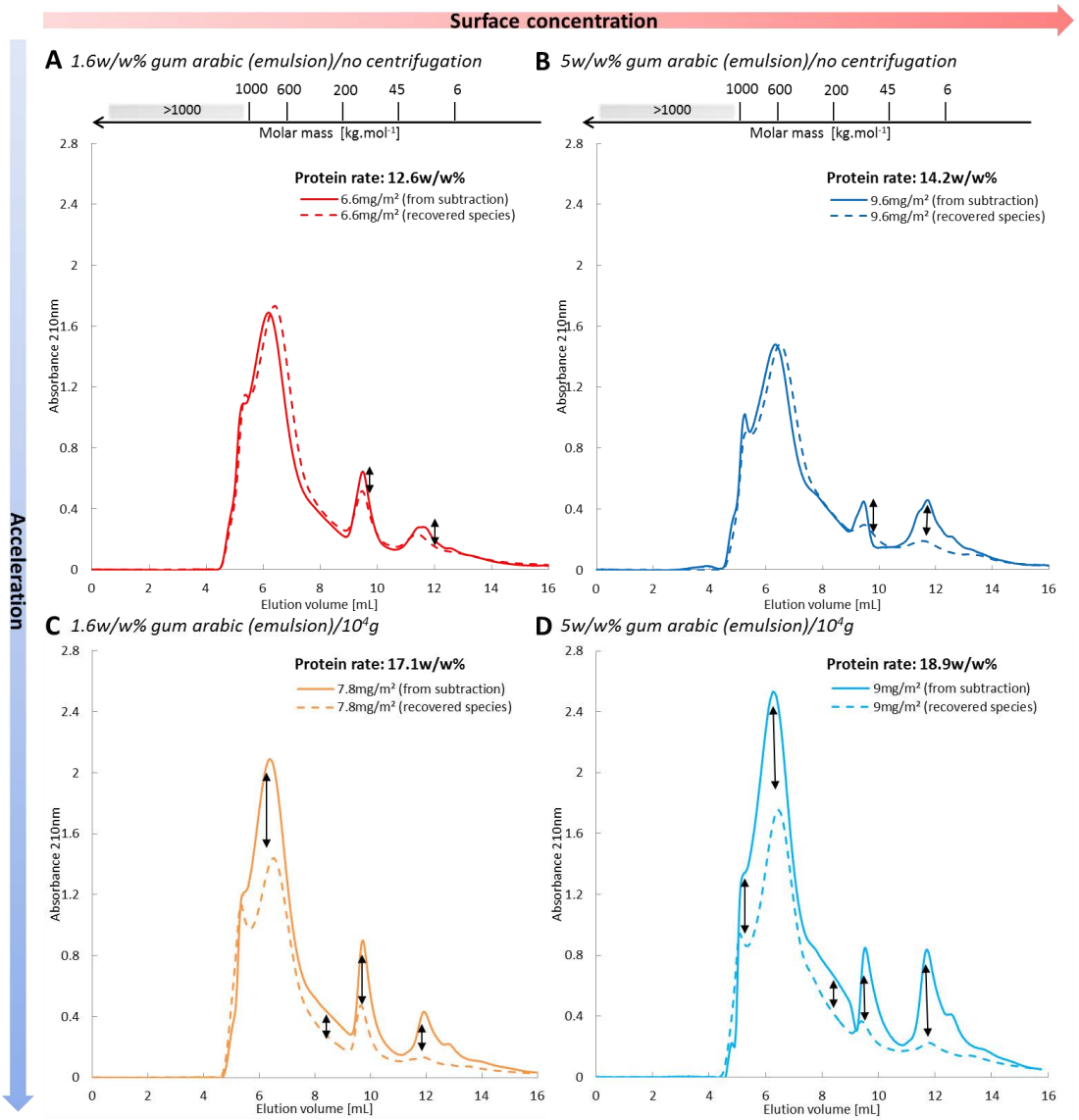

Figure IV-4: Comparison of adsorbed species size exclusion chromatograms (molecular weight distribution) Calculated chromatograms (continuous line) and recovered species chromatogram (dotted line). (A) Emulsion stabilized with a 1.6w/w% GA solution and no centrifugation, (B) Emulsion stabilized with a 5w/w% GA solution and no centrifugation, (C) Emulsion stabilized with a 1.6w/w% GA solution, centrifuged at 10^4 g, (D) Emulsion stabilized with a 5w/w% GA, centrifuged at 10^4 g.

UV detection signal at 210nm being mostly sensitive to the peptide bonds [18], [19], area under the calculated chromatograms of the interfacial film (continuous line) is proportional to the interface protein rate, which is given for each case investigated. The order of magnitude of the protein rate at the interface is 16%w/w (between 12.6 to 18.9 w/w%), compared to the 2.9% in the native gum, illustrating the contribution of the gum polypeptide moieties in adsorption process.

In the case of emulsion prepared with the lowest gum Arabic concentration (1.6 w/w%) and without centrifugation (Figure IV-4A), a weak difference between both chromatograms is observed for the smaller size macromolecules eluted around 9.5 and 11.5 mL (6-200 $kg.mol^{-1}$). A similar behavior can be noticed for the emulsion stabilized with a 5w/w% gum Arabic solution and without centrifugation (Figure IV-4B): smaller macromolecules preferentially aggregate. Comparison between Figure IV-4A and B shows that the aggregation rate is increasing as the surface coverage and protein content increases (black arrows on the graphs).

When these emulsions were submitted to centrifugation during the rinsing steps, the composition of the interfacial film changed with a noticeable increase of the protein rate. Figure IV-4C and D show that the aggregation at the interface concerns almost all the range of size spectrum of macromolecules. With centrifugation and increase of the surface concentration, high molecular masses (elution volume between 4.5 and 7mL) (>1000-600$kg.mol^{-1}$) and intermediary (elution volume between 7 and 9.5mL) (600-200$kg.mol^{-1}$) also aggregate. As in the case with no centrifugation, comparison between Figure IV-4C and 4D discloses an increase of aggregation rate for each class of molecular weight when the surface concentration is increased (between 1.6 and 5 w/w%), in particular for the molecules eluted at 5mL and 9.5mL (Figure IV-4D).

As indicated by the protein rate, the global hydrophobicity of the interface increases when more species aggregates. Moreover we observed that intermediary molecular weight macromolecules desorb when coalescence events occur. In a former study, we have demonstrated that the interface (of emulsions that were not centrifuged) was preferentially crowded by smaller macromolecules of the gum (6-200 $kg.mol^{-1}$). These ones being more numerous at the interface, it is then plausible that they preferentially aggregate to structure the interfacial film. However, whereas the composition of the interface is changing due to the desorption of the less hydrophobic moieties (intermediary molecular masses) the aggregation process concerns almost the whole range of adsorbed moieties. An increase of the surface concentration not only induces an increase of the protein rate but also an increase of the aggregation rate between adsorbed species. These observations contrast with the view of the gum high molecular weight protein-polysaccharides conjugates (AGP) as the main contributors to the emulsion stabilization by steric repulsion. In return, the question of the relationship between this observed structuration of the interface and the emulsion metastability seems justified.

What is the structure of the aggregates?

Given the colloidal nature of gum Arabic species, small-angle scattering methods are well-suited to their non-intrusive characterization. The colloidal length scales can be studies through X-ray or neutron small-angle scattering (SAXS and SANS). For isotropic samples, the structure is described in

the reciprocal space through scattered intensity versus q spectra, with q the magnitude of the scattering vector, expressed in reverse distances. The low q range thus corresponds to large correlation length scales while the high q range corresponds to small correlation length scales. An important difference between these two similar methods are the origin of contrast, which relates to electronic clouds for SAXS and to nucleus types for SANS. Notably, the use of deuterated molecules is a convenient way to tune the SANS contrast. We have used both SAXS and SANS to characterize gum Arabic in solution in another work and sum up here the main results of interest to the present study.

SAXS spectra are dominated by a structure correlation peak when the gum is dissolved in pure water. This correlation peak associates with a correlation distance that scales with the cubic root of gum concentration, indicating repulsions between well-defined objects. This distance is of 44 nm for 0.5% solutions and 21 nm for 5% solutions. From a 20 mM concentration in NaCl, the ionic strength is sufficient to screen ionic repulsions, which results in the disappearance of the structure peak. The scattered intensity reaches a plateau at low q values, which corresponds to a finite object possessing a gyration radius of around 7 nm. At higher q value, whatever the concentration in the 0.5-5% range or the ionic strength, two oscillations are observed, which correspond to structures smaller than the species responsible for ionic repulsions.

SANS spectra yield similar results at intermediate and high q values but drastically different ones at low q values. An intensity upturn is observed, with a q^{-1} followed by q^{-3} at 0.5% and a q^{-2} at 5%, independently of ionic strength. This SANS/SAXS mismatch is uncommon and calls for an explanation. We verified that SAXS spectra of both H_2O gum solutions and D_2O gum solutions were identical. The SANS/SAXS mismatch is thus unrelated to any slight interaction differences in the two systems, which may manifest in the vicinity of a phase transition. This leaves only another explanation related to differences in contrast distribution within the scattering material. This corresponds to a heterogeneous scattering system, the parts of which display important contrast differences with the solvent, between SAXS and SANS experiments. SANS experiments were performed by dissolving gum Arabic in deuterated water (D_2O) so that the contrast difference mainly originates from the hydrogen/deuterium ratio, while in SAXS experiments the contrast difference originates from differences in electron density. SANS/SAXS mismatch likely results from two superimposing phenomena, which both highlight the polypeptide backbone compared to the polysaccharides. Firstly, hydrogen-deuterium exchange is expected on labile hydrogen [20], which are present on both hydroxyl (sugars) and amine groups (proteins). This will lead to a contrast decrease between polypeptide/polysaccharides and deuterated water, which will be more pronounced for the polysaccharides since they contain more hydroxyl moieties. Secondly, polysaccharides are likely more hydrated than at least some parts of the polypeptide chains, which possess hydrophobic amino-acids and secondary structures. This will drastically reduce the contrast of polysaccharides except at

molecular length scales, which probe directly hydration. We can thus conclude that the intensity upturn in the low-*q* range indicates large scale structures of inner parts of the gum structure, which are likely polypeptide chains.

At low gum concentrations, we proposed a structure in solution consisting of protein rich clusters co-existing with protein-poor regions. Inside these clusters, the chains would be locally extended yielding the q^{-1} slope. At high gum concentrations, we argued in favor of interpenetration of these clusters, since it is close to overlap, which leads to more compact structures at intermediate length scales but an overall decreased compacity at large length scales. We now turn towards a comparative structural characterization of solutions containing species collected from oil/water interfaces.

Figure IV-5A and B respectively display the SAXS and SANS spectra of aqueous solutions of both gum Arabic and adsorbed species recovered from emulsions stabilized with a 5w/w% gum Arabic solution with and without centrifugation. In the three cases, the mass concentration of gum species is 0.5% and the concentration of NaCl is 0.02M. With both SAXS and SANS techniques, we observe a large increase of the scattered intensity at low *q* values (large length scales), from gum Arabic spectra to spectra of adsorbed species recovered after centrifugation. This increase in scattering intensity is consistent with the corresponding increase of turbidity observed in Figure IV-3. However, the intensity scattered by a given sample significantly differs in this low *q*-range between SANS and SAXS spectra while it is identical in the high *q*-range. It is higher in SANS than in SAXS spectra and follows a different power law. As discussed above, SANS spectra highlight polypeptides while SAXS spectra represent all species in their relative proportions, which favors polysaccharides. Differences in power law are thus indicative of structural differences between the two types of species.

On the gum Arabic SAXS spectra, a finite size is observed at low *q* values with the scattered intensity reaching a plateau value, whereas a q^{-3} slope is observed on the SANS spectrum, corresponding to collapsed chains. On the adsorbed species spectra, both SAXS and SANS spectra indicate that no finite size is observed over the probed length scale. However, for the adsorbed species from the centrifuged emulsion, the SAXS curve yields a $q^{-1.45}$ slope while the SANS curve yields a $q^{-2.75}$ slope. The SAXS slope indicates a swelled structure in a good solvent, which is consistent with hydrophilic polysaccharides chains. The SANS slope suggests a fairly collapsed structure in a bad solvent, in accordance with the signature of hydrophobic polypeptide chains. This difference shows that both hydrophilic and hydrophobic moieties optimize their conformation in solution, resulting in a difference in mesoscopic organization. Like native species, the aggregates are rather porous than dense.

Since the signal-to-noise ratio was much better on the SAXS than on the SANS spectra, a closer examination of the scattering behavior at high *q* values is possible. Figure IV-5D displays the SAXS spectra in a Kratky plot ($I(q)q^2$ vs. q), commonly used to investigate intrinsically disordered proteins [21], [22]. The area under the curve is proportional, for a given contrast, to the total volume of the scattering material. Three oscillations are visible on the spectrum of gum Arabic at 0.246, 0.854 and 2.54 nm^{-1}. We showed in our study of the gum in solution that the first oscillation corresponds to polysaccharides moieties (Arabinogalactan-peptide) with a 7nm gyration radius. The two other oscillations correspond to smaller structures with gyration radius $3^{1/2}/q$ of 2 and 0.7 nm and characteristic distances of $2\pi/q$ of 7 and 2.4 nm. In a previous study we showed that these oscillations were possibly the signature of the presence of several populations with the gum.

A striking observation is the SAXS spectra of adsorbed species is the intensity collapse at these small length scales, which counterbalances the intensity gain observed in the low *q* region. Furthermore, both the first and last oscillations remain but the intermediate oscillation is nearly absent on the spectra of adsorbed species collected from a non centrifuged emulsion and absent for species collected from a centrifuged emulsion. This second oscillation originate from the second population of arabinogalactan-peptide (AGp2) within the gum. Knowing from Figure IV-4 that this population preferentially aggregate at the interface, the loss of this oscillation might originate from the loss of the signal of AGp2 due to irreversible aggregation. Moreover, circular dichroism measurements were performed on the same sample of recovered species (see Figure IV-14 in supporting information), and results indicates that the secondary structure of the protein moieties was not lost (optical activity intact) when recovery species from the interface from the separation procedure used. This observation further support the hypothesis of the oscillations observed in SAXS measurements coming from several population and not secondary structures.

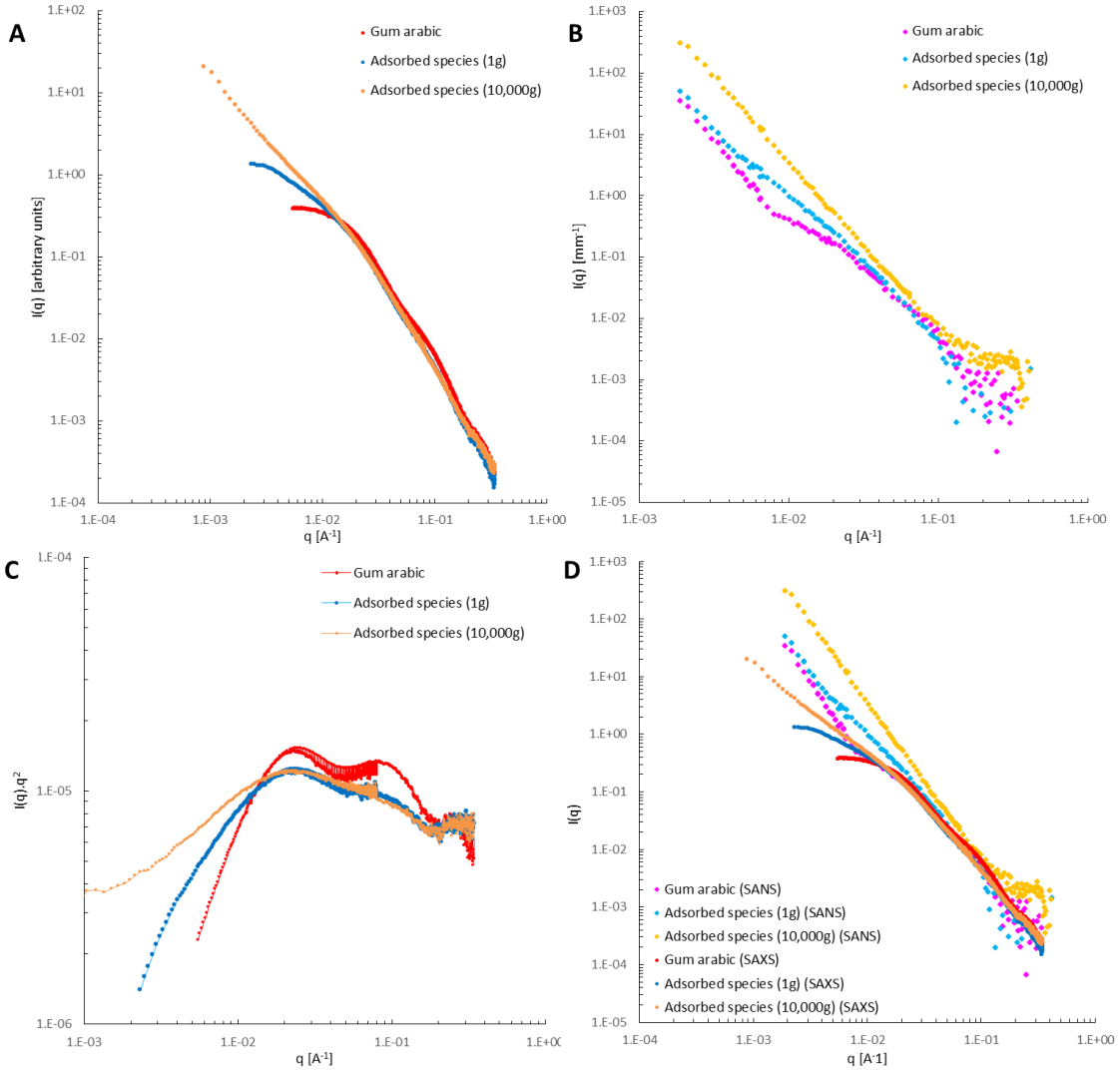

Figure IV-5: Small angle scattering spectra of gum Arabic and adsorbed species in solution at 0.5w/w% in 20mM NaCl (A)X-ray scattering, (B)Neutron scattering (samples are dissolved in D_2O), (C) Kratky-plot type, (D) Superposition of SANS(diamond shape) and SAXS data (round).

How does interfacial aggregation take place?

So far interfacial aggregation mechanism has been evidenced using indirect bulk characterizations of gum species before and after emulsification. We now turn towards more direct measurements of the oil/water interface in gum stabilized emulsions using SANS. In a full contrast, such as deuterated water/hydrogenated oil, the main quantities that can be characterized from SANS spectra relate to the interfacial area, the interface smoothness and the characteristic size of water films separating droplets in a concentrated emulsion. These quantities are all linked to the extend of the Porod regime for which $I(q)$ varies as q^{-4}, and mask the signal of adsorbed species. However, the contrast can be drastically modified to visualize only the hydrogenated adsorbed species working with

deuterated oil at the same neutron scattering length density as deuterated water. In these contrast-matched conditions, oil/water interfaces are invisible and the scattered intensity only originates from the adsorbed species since the cream is thoroughly washed with pure D_2O prior to measurement. Two types of emulsions with contrasted amount of gum have been prepared with different emulsifying devices so that one corresponds to a much larger gum/interface ratio than the other. They are designated below as dilute and concentrated samples. The resulting SANS spectra are displayed in Figure IV-6 together with two SANS spectra of gum Arabic in solution at respectively 0.5% and 5%. The two spectra of adsorbed species (purple and blue symbols) are relatively similar in the high q range and a finite length scale of a few nanometers is observed. In the concentrated sample a q^{-1} slope is observed, which suggests one dimensional structures. In the dilute sample, the intensity scales with $q^{-0.4}$, which rather suggests nearly isolated objects with a few linkages. In the high range, large differences are observed with a q^{-1} followed by q^{-3} scaling for the dilute sample and a q^{-2} scaling for the concentrated sample.

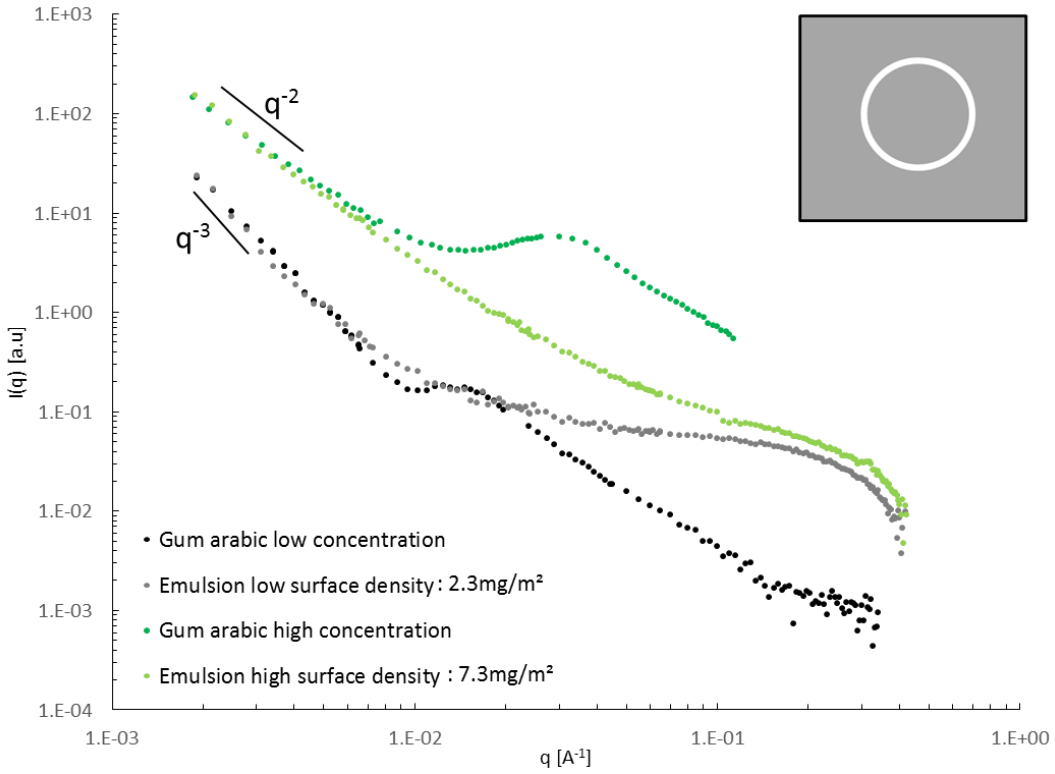

Figure IV-6: Small angle neutron scattering spectra of GA adsorbed films (blue and purple symbols) in emulsions in contrast-matched conditions compared to D_2O solutions spectra (red and green symbols) at different gum concentrations. The spectrum for gum Arabic at 5w/w% comes from Dror et al, 2006 [23].

In Figure IV-6, the SANS spectra of the two GA deuterated solutions at 0.5 and 5 w/w% have been reported (red and green symbols respectively). The scattered intensity of the concentrated sample is much higher than the dilute sample, which indicates that more species are adsorbed at the oil/water interface. This difference would be even higher if the curves were renormalized by the total

interfacial area. Indeed, the two emulsions were prepared using rather different emulsifying devices, which lead to different size distributions. Using the Porod law for similar but fully contrasted emulsions shows that the emulsion prepared with less gum Arabic has three times more interfacial area than the emulsion prepared with more gum Arabic.

The low-q range behavior of the concentrated sample in $q^{-2.2}$ closely matches that of the 5% GA solution in q^{-2}, while the q^{-3} behavior of the dilute sample matches that of the 0.5% GA solution. We can thus conclude that the structure of adsorbed species at large length scales matches their structure in solution at the relevant concentration. We can also conclude that significantly different packings are obtained upon varying the gum/area ratio.

Over the rest of the q-range, significant differences appear. In the intermediate q-range, the correlation peak corresponding to ionic repulsions between charged polysaccharides moieties is clearly visible on the GA solution spectra. Such a peak is absent in both emulsions spectra. It can thus be stated that adsorbed species must adopt a conformation that does not optimize ionic repulsions. This leads to the conclusion that the conformation is optimized with respect to another interaction. We have shown in our work on the interface composition that the protein rate of adsorbed species increases compared to the native gum, which suggests that polypeptides are indeed responsible for adsorption at oil/water interfaces. Microscopically, this adsorption could proceed through hydrophobic interactions between the oil phase and the hydrophobic amino-acids of the polypeptide chains or through conformational changes such as denaturation of secondary structures. We have observed that extensive centrifugation of emulsions could result in a partial desorption of some species with an intermediate hydrophobicity and limited hydrophobic amino-acid content compared to the rest of polypeptide chains. This suggests that the strength of adsorption is indeed related to the hydrophobic amino-acid content. The scattering spectra on droplets' interfaces suggest that at low surface concentrations small nanometric objects are present with limited linkage, while at large surface concentrations these objects seem to organize unidimensional objects. This organization could suggest train-like arrangements, which was suggested by Snowden et al. using electron spin resonance spectroscopy.

We can conclude from these experiments that the conformation of gum Arabic species dramatically changes at small and intermediate length scales but remains similar to the conformation in solution at larger length scales. At all length scales, gum concentration is a crucial parameter. Interestingly, we demonstrated that the gum concentration plays only moderately to the composition of the oil/water interface but drastically to the coverage density. Increasing the concentration leads to significant increase of the mass adsorbed per unit area. This increase is consistent with the changes observed the emulsions SANS spectra both in overall scattered intensity and characteristic slopes compared to the solution spectra. Interfacial aggregation is promoted likely through contact between

hydrophobic clusters that were otherwise shielded from each other in solution by steric/ionic repulsions. When adsorbed species are collected and dissolved in solution, the conformation partially relaxes at high q, which leads to significant changes also at low q. This conformation irreversibility is the hallmark of strong aggregation.

Conclusion.

Gum Arabic, a mixture of covalently linked proteins and polysaccharides, is extensively used for its stabilizing properties of oil-in-water emulsions. In the literature the high molecular weight polysaccharide-protein conjugates from the gum (AGP) are described as the emulsifying species adsorbing at oil droplet interfaces providing strong steric repulsions and responsible for the stabilizing mechanism. Here, we have provided direct evidence for the formation of a hydrophobic network at the oil/water interface in gum Arabic stabilized emulsions. The structure of this network echoes the structure of gum Arabic solutions at the corresponding concentration at large length scales but presents significant differences at small length scales. Notably the spectra suggest isolated to unidimensional assemblies of nanometric objects upon increasing the surface concentration. Only a partial conformational relaxation occurs when dissolving anew adsorbed species in solution, which demonstrates that irreversible aggregation occurs as a result of these structural changes at small length scales. We obtained the composition of these aggregates as a function of the various conditions and demonstrated that they were of glycoproteins and a small hydrophobic population of Arabinogalactan peptide at low surface densities. At higher surface densities, large Arabinogalactan-protein conjugates also enter the aggregate composition. Centrifugating emulsions to reduce their interfacial area results in the partial desorption of the small Arabinogalactan peptides and a drastically extended aggregation of all species composing the film. We suggest that the metastability of gum Arabic stabilized emulsions, which can be excellent or rather poor, mostly stems from the formation of this network rather than steric/ionic repulsions between polysaccharides.

Materials and methods.

Materials

Powder gum arabic (Acacia senegal) was a gift from Caragum International ® (Marseille, France) and the composition was 2.9% of proteins (Nx6.25), 11% moisture, 3.16% ash and nearly no lipid. Sodium chloride (99.5>% BioXtra), hexadecane (99% ReagentPlus), pentane and hydrochloride acid were purchased from Sigma Aldrich. Distilled water was used for all experiments.

Emulsion preparation

Oil-in-water emulsions stabilized by gum arabic were prepared using a rotor/stator device (Ultra-Turrax ®). Alkane oil (pentane or hexadecane) were used as the organic phase. We showed that the nature of the oil did not influence the composition of the interface. Emulsions were formulated as follow: 20 or 40v/v% of oil was dispersed in gum Arabic aqueous solution. Gum Arabic solutions were prepared at different concentrations and centrifuged under 19000g to remove insoluble materials. Then the pH or the salt concentration of each solution was adjusted by adding small amounts of HCl at 1mol.L^{-1} or NaCl at 3 mol.L^{-1}. Both phases were mixed during one minute at 10000rpm (for emulsions with turrax) or 15000rpm during 3 minutes (for emulsions with dispax).

Drop size distribution

Droplet size distributions of emulsions were measured using a static light scattering instrument (Mastersizer 3000, Malvern ®). The laser wavelength was of 633nm. An emulsion sample was dispersed in recirculated water until at an obscuration rate between 1-10% was obtained to avoid multiple scattering. The average droplet diameter was determined as the volume-surface mean diameter d[3,2]. Each measurement was repeated in triplicate at room temperature.

Interfacial separation

Gum arabic amphiphilic species were separated through an emulsion mediated separation technique. Emulsions of pentane in water stabilized by Arabic gum were formed following the aforementioned protocol. Emulsions were then placed in a separative funnel, diluted with distilled water saturated with pentane (in order to avoid pentane to diffuse from oil droplets into the aqueous phase) and let to cream or centrifuged. In both case the aqueous phase was recovered and the emulsion cream was rinsed again in order to remove all species that could be trapped between droplets without being strongly adsorbed at an interface. The aqueous phase containing all the species that did not adsorb was then dialyzed against water and lyophilized. The final cream was dispersed in water and freeze-dried.

A mass balance on the recovered species was carried out on each batch in order to calculate the surface concentration of the droplets.

Interfacial film composition

The surface cencentration (in mg.m^{-2}) in emulsions was determined using equation IV-1. The amount of recovered mass (m_{ads}) was determined from the mass balance from the interfacial separation procedure.

$$\Gamma = \frac{m_{ads} d_{[3,2]}}{6V_{oil}} \quad \text{(IV-1)}$$

Chromatograms of adsorbed species at interfaces were determined using the following procedure: a native gum Arabic sample and the species recovered from the aqueous phase of the emulsion (non adsorbed species) were solubilized in water at a concentration of 3w/w%. The same volume for each solution was injected into both chromatographic columns (SEC and HIC). For both chromatographic techniques, the total area of chromatograms has been found proportional to the species concentration of each sample. Knowing from the interfacial separation procedure the mass percentage of macromolecules from the gum that did or did not adsorbed, the chromatogram of the species recovered from the aqueous phase was multiplied by its mass percentage. In order to obtain the chromatogram of the adsorbed species the chromatogram of the non adsorbed species was subtracted to that of the gum Arabic. Finally in order to compare each chromatogram between them, the resulting chromatogram was multiplied by one hundred divided by its mass percentage determined from the interfacial separation procedure (percentage of gum macromolecules that adsorbed from the amount of gum used in the emulsion).

Nitrogen rate fraction analysis

Nitrogen rate analyses were performed at the Laboratoire de Coordination Chimique (Toulouse, France). A Perkin-Elmer 2400 CHN series II was operated at 1050 °C with oxygen as carrier gas. Each sample was analyzed in duplicate. Protein fraction rate was deduced from the nitrogen rate through the protein conversion number calculated from the gum Arabic proteins amino acid distribution (Renard et al., 2006).

$$\%_{protein} = \%_{nitrogen} \times 8.14 \qquad \text{(IV-2)}$$

Transmittance measurements

Solutions transmittances were measured using a UV-visible spectrophotometer between 200 and 800nm. Quartz cells were used and the background was performed using a cell filled with distilled water.

Size exclusion chromatography

Size exclusion chromatography was used to separate Arabic gum species as a function of their relative hydrodynamic volume. A 7.8mmx300mm BioSuite 450Å SEC column (Waters ®) packed with 8µm porous silica beads was used. The average pore size of the silica beads is 450Å.

The separation was performed on an Alliance HPLC unit (Waters 2695 separations module), a 0.5mol.L^{-1} NaCl aqueous solution at 25°C was used as the eluent phase at a flow rate of 0.8mL.min^{-1}. Each sample was filtrated with a nylon 0.2µm membrane and a volume of 50µL was injected. UV detection was performed at 210 nm and 280nm using a Waters® 2487 UV detector. A refractive

index detection was used to measure the mass percentage of each eluted moity (Waters® 410 differential refractometer). The refractive index detection was only available until 10.5mL of elution due to a negative peak appearing from the difference in refractive index between the sodium chloride of the eluent phase and the water from the injected sample. The column was calibrated using branched dextran standards (Waters ®). [24]

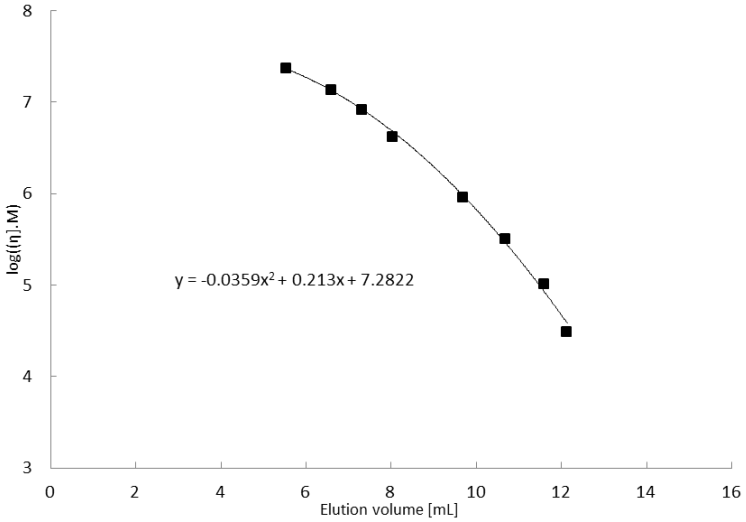

Figure IV-7: Universal calibration curve (realized with branched dextran of known molecular weight) for size exclusion separation on Biosuite 450Å SEC column (waters) with a low rate of 0.8mL/min and 0.5M NaCl as the eluant solution.

Variation coefficients for each detection were calculated from three injections of the same sample. For UV detection at 210nm a 0.3% variation coefficient was measured. It was of 0.5% for UV detection at 280nm and of 13% for the refractive index detection.

UV detection at 280nm is more sensitive to the rate of cyclic amino acid present within the gum polypeptidic moieties (tyrosin and phenylalanine). Cyclic amino acids however are not the predominant amino acid moiety in gum Arabic, as already observed in the literature (Goodrum et al., 2000; Randall et al., 1989; Ray et al., 1995). UV detection at 210 nm is mostly sensitive to $\pi \rightarrow \pi^*$ transitions from C=O bond of the amides linkages between amino acid moieties, and from those of carboxylic moieties present onto the polysaccharides backbones and of reducing sugar units at the end of polysaccharides chains.

Hydrophobic interaction chromatography

Hydrophobic interaction chromatography was used to separate Arabic gum fractions as a function of their hydrophobic properties. A 7.5mmx75mm 10µm Biosuite Phenyl column (Waters ®) which consist of phenyl groups bonded to a metacrylic ester based polymeric resin. The average pore size of the column is 1000Å to accommodate macromolecules with high molecular weight. A gradient of

salt concentration is required to create a "salting out" effect and progressively desorb the hydrophobic species. The less hydrophobic species are eluted first with highest salt concentration, while the more hydrophobic species are the last one eluted.

The separation was performed on an Alliance HPLC unit (Waters 2695 ® separations module). The mobile phase was composed of a solution of NaCl at a constant flowrate of 0.5mL.min^{-1}. A continuous time gradient of salt concentration between 4mol.L^{-1} and 0 was applied during 22minutes followed by distilled water during 12 more minutes (Figure IV-8). Each sample was filtrated with a nylon 0.2µm membrane and a volume of 30µL was injected. UV absorbance was measured at 210 nm and 280nm (Waters® 2487 UV detector). We observed that UV absorbance (especially at 210nm) was very sensitive to NaCl concentration. The baseline was not constant along the separation time. In order to correct this deviation the chromatogram of distilled water injected in same elution conditions was subtracted to each measured chromatogram.

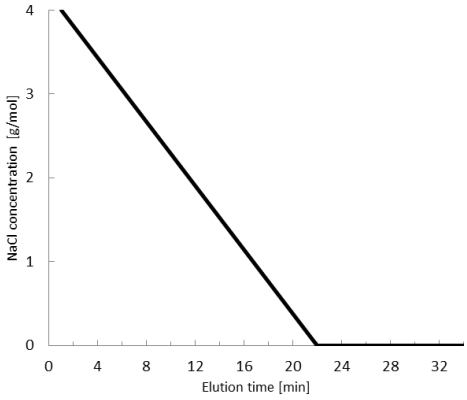

Figure IV-8: NaCl elution gradient used for hydrophobic interaction chromatographic separation.

Variation coefficients for each detection were estimated from comparison of three injections of the same sample. A 6% (resp. 11%) variation coefficient was observed at 210nm (resp. 280nm).

Small angle neutron scattering

SANS experiments were performed on the PAXY small angle diffractometer located at the Orphee reactor in Saclay, France. Samples were dissolved in 20mM NaCl solution in D_2O. Spetra were recorded using four different spectrometer configurations: λ=6Å (incident wavelength), d=1m (distance of the sample to the detector), λ=6Å and d=3m, λ=8.5Å and d=5m and λ=15Å and d=6.7m. The range of wave vectors q covered was from 6.5.10^{-3} to 4.5.10^{-2} Å^{-1}. The data set was normalized for transmission and sample path length. A subtraction of the incoherent background was performed using the sample transmission at high q values and a calibration of D_2O/H_2O solutions at different ratios (transmission as a function of scattered intensity).

Small angle X-ray scattering

SAXS experiments were performed on the ID02 instrument at the ESRF synchrotron facility in Grenoble, France. Samples were prepared using 1.5 mm disposable quartz capillaries. Measurements were taken at 1.5 and 7m. Azimuthal averaging was performed after mask subtraction and data was normalized by the transmission. One dimensional spectra were subtracted by the capillary filed with water or the buffer solution.

Circular dichroism

Circular dichroism measurements were performed on gum Arabic and gum SEC fraction on a Jasco J-815 CD spectrometer at IMRCP laboratory in Toulouse, France. A quartz cell with a 10mm path length was used. Measurements were performed between 250 and 185nm at 25°C. A concentration of 0.025% was used for gum Arabic and of 0.00625% for FA, FB and FC. FD was not measured due to its low solubility. Results were normalized by the concentration and the path length to be comparable.

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Supporting information.

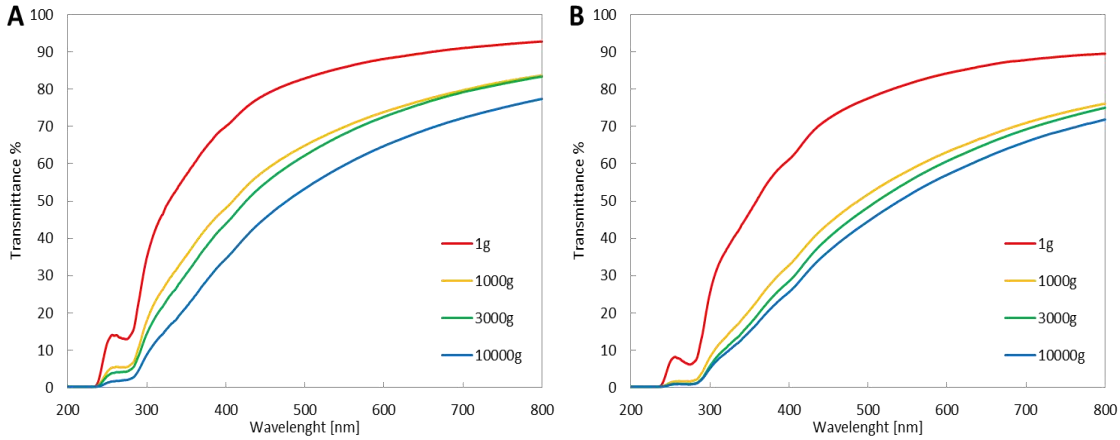

Figure IV-9: Transmittance of adsorbed species solution (1w/w%) between 200 and 800 nm for various centrifugals acceleration applied during rising steps (1g corresponds to the case without centrifugation). (A) Emulsion stabilized with a 1.6w/w% GA solution, (B) Emulsion stabilized with a 5w/w% GA solution.

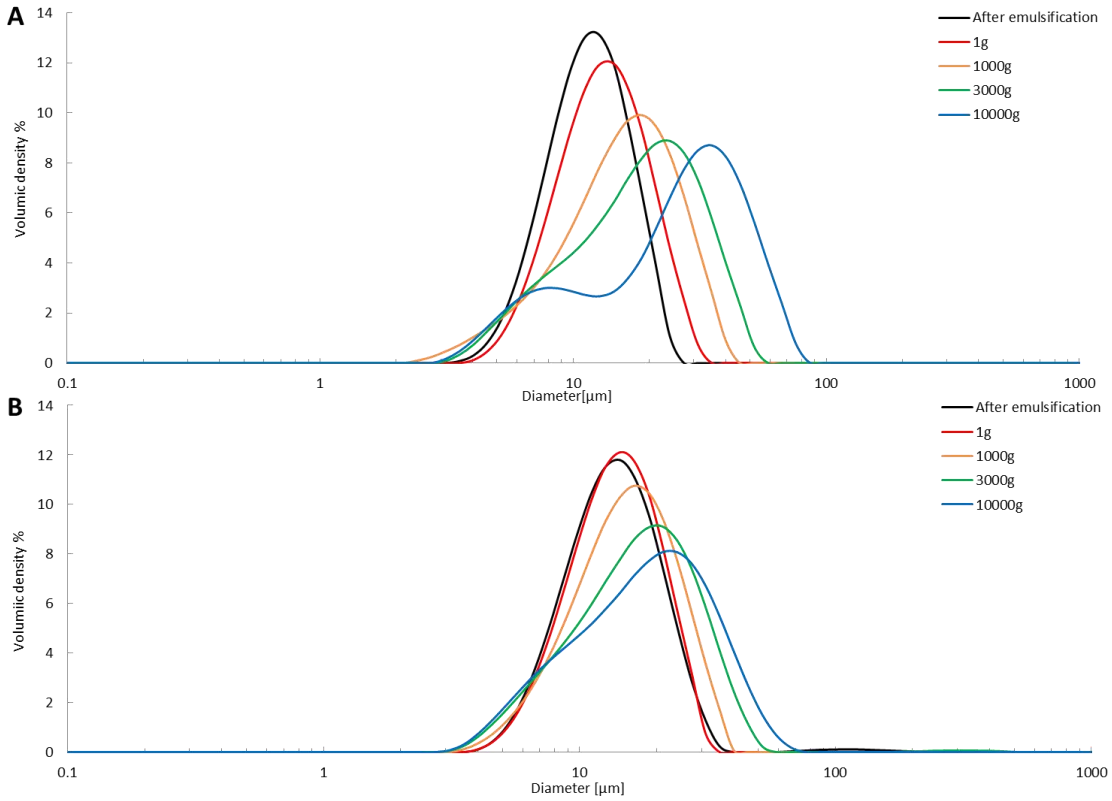

Figure IV-10: Evolution of droplet size distribution as a function of centrifugation acceleration: (A)emulsion stabilized with a 1.6w/w% GA solution and (B) emulsion stabilized with a 5w/w% GA solution.

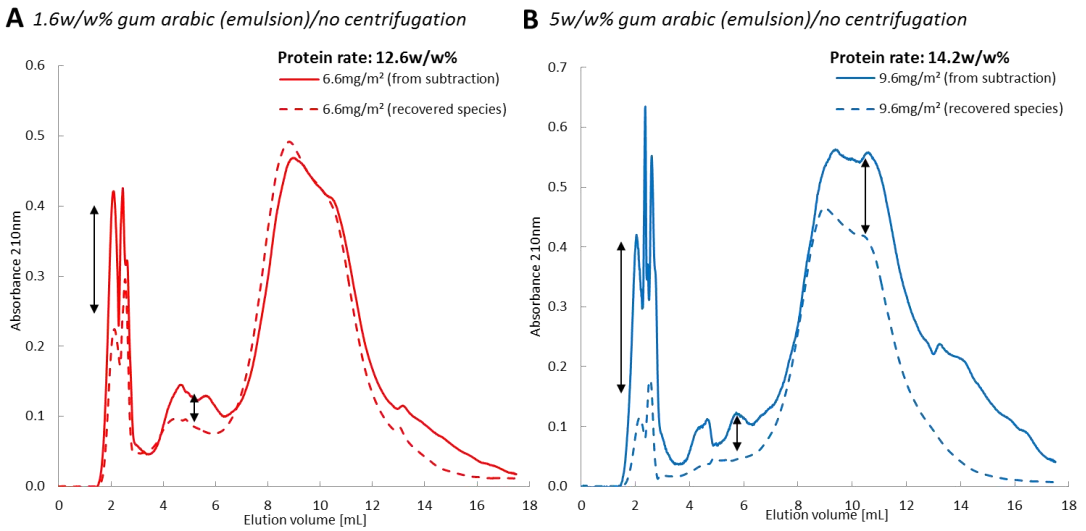

Figure IV-11: Hydrophobic interaction chromatograms: comparaison of adsorbed species calculated spectrum (from subtraction of non adsorbed species chromatogram to GA chromatogram) and recovered adsorbed species spectrum. (A) Emulsion stabilized with 1.6w/w% GA solution, (B) Emulsion stabilized with 5w/w% GA solution.

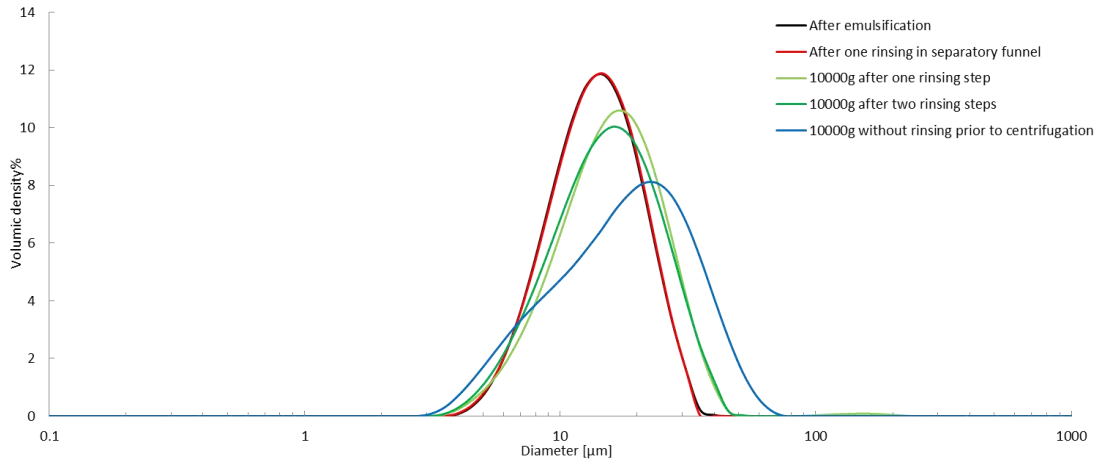

Figure IV-12: Evolution of GA stabilized emulsion (5w/w%) size distribution for different conditions of the adsorbed species recovering process.

From *Figure IV-12* we observed that a GA stabilized emulsion better resisted to centrifugation when almost all the non-adsorbed species were removed from the aqueous phase (by a rinsing step in a separating funnel prior to centrifugation). Therefore more coalescence events occur when droplets are packed in the presence of non-adsorbed macromolecules. This observation tends to prove that non-adsorbed macromolecules of the aqueous phase not only do not favor droplet stabilization, but on the contrary promote coalescence. This result is interpreted as follows: when droplets are bring into contact, the adsorbed layer is compressed. In the presence of macromolecules in the interstitial

aqueous film, less water will be available to hydrate the adsorbed moieties and thus droplets will be less protected against film rupture.

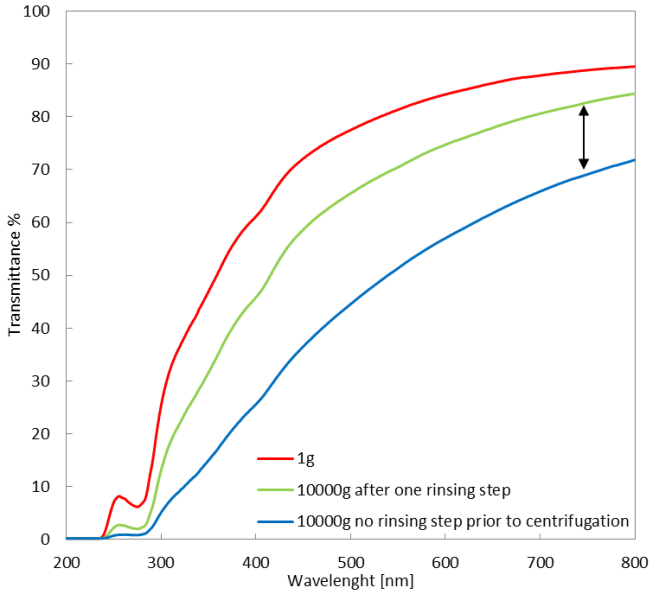

Figure IV-13: Transmittance of adsorbed species solution (1w/w%) between 200 and 800 nm at different conditions of the interface species recovering process. (Emulsion stabilized with a 5 w/w% GA solution).

Measurement of recovered species solution transmittance revealed that they were less aggregated when removing the non-adsorbed species from the aqueous phase prior to centrifugation as can be seen in *Figure IV-13*. This result is in agreement with the observation of the size distribution evolution of *Figure IV-12*. Species must be less concentrated at the interface then in the case of the non rinsed emulsion centrifuged at 10^4g.

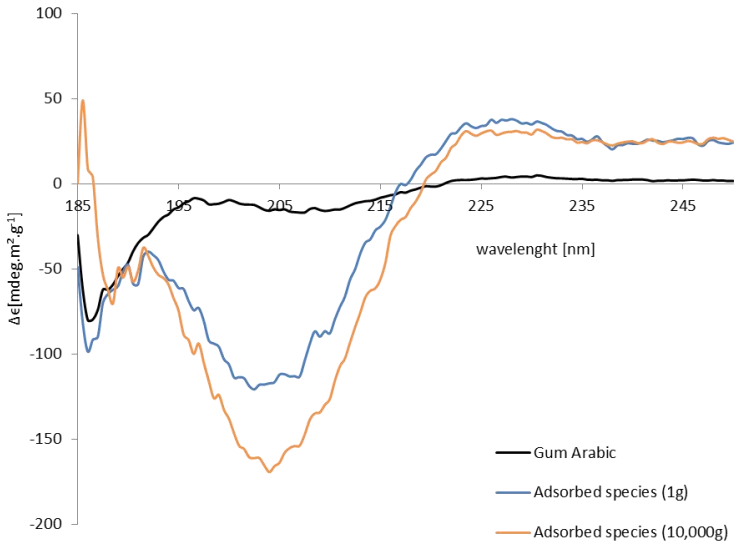

Figure IV-14: Circular dichroism measurements of Arabic gum and recovered species from the interface. Results were normalized by the cell path length and the sample concentration.

Conclusion and perspective

Understanding the stabilization of emulsions by amphiphilic hydrocolloids combines several challenges:

- Hydrocolloids like gum Arabic are themselves composed of several distinct colloidal entities, each possessing a multi-scale structure and capable to self-associate in solution.
- The composition of interfaces in equilibrium with aqueous solutions is expected to drastically differ from bulk composition in complex mixtures. The bulk/interface relationship may be either thermodynamically or kinetically controlled, which associates to an important impact of formulation conditions.
- Emulsions are non-equilibrium systems the metastability of which is controlled by the dynamic interfacial structure, which relates to but differ from the static structure.

This thesis work addressed these different issues in order to gain a better comprehension of the emulsifying properties of gum Arabic.

In the first manuscript displayed in chapter 2, we presented a study on the structural composition of gum Arabic species in solution. Combining new experimental results with relevant previously published studies, we came to important clarifications regarding the structure of gum Arabic in solution. A two-dimensional separation of gum Arabic species, using size exclusion as the first dimension followed by hydrophobic interaction, was achieved and compared with another dual chromatographic separation method taken from the literature by Renard and coworkers. We concluded that neither size (molecular weight) nor hydrophobicity are truly efficient separation criteria. Nevertheless, combining both methods together with structural characterization by small-angle scattering, yields a more accurate overview of the various species composing gum Arabic and of their association in solution. This description is consistent with literature reports, but more specific. Two populations of arabinogalactan-peptide (AGp) were identified, rather than one. They differ in size, hydrophobicity and protein content but correspond to an equal number of species. Four populations of glycoproteins, rather than one, corresponding to different sizes and content in hydrophobic amino-acids were also identified. Larger gum species are made up of a combination of these two populations to form arabinogalactan-protein conjugates (AGP), underlying the great heterogeneity in size, even after enzymatic or chemical attacks. In solution, the structure is dominated at large length scales by polypeptide association, which drastically changes with gum concentration from a cluster regime to a semi-dilute regime. At smaller length scales, the signature of several porous structures is observed, one of them corresponding to hyper-branched and weakly charged polysaccharides present in the AGp populations. Smaller structures are detected, possible signature of secondary structures and hydrophobic clusters.

Gum Arabic adsorbed layers in oil-in-water emulsions were considered in the second manuscript presented in chapter 3. The literature being less abundant on this topic, a more extensive characterization work was requested. We first developed a method to probe interfacial film composition using both size exclusion and hydrophobic chromatography, which relies on the irreversible nature of gum species adsorption at oil/water interfaces. These interfaces were shown to be enriched in protein-rich species from the gum, which display a broad range of sizes. This result differs from the commonly accepted view that only the high molecular weight conjugates of the gum are of importance regarding adsorption process and emulsion metastability. We observed that surface concentrations of gum Arabic stabilized emulsions were highly dependent on the emulsions formulation (gum concentration, pH and ionic strength): decreasing ionic repulsions through an increase of the solution ionic strength or a decrease of the pH led to an increase of the mass adsorbed per unit area. Interestingly, this surface concentration change corresponded to only minor changes in the composition of the adsorbed layers, indicating that adsorbed macromolecules from gum Arabic may undergo significant conformation changes at an oil-water interface compared to their conformation in solution.

Chapter 4 focuses on the stabilization mechanisms at play when using gum arabic in oil-in-water emulsions. First, the metastability of gum Arabic stabilized emulsions was observed and it was shown that, depending on the formulation, emulsions exhibited either an outstanding resistance to destabilization processes or a poor metastability. Recovery of gum Arabic adsorbing species surprisingly evidenced the presence of aggregated moieties (cloudy solution), the intensity of which depended on emulsification conditions (surface concentration and interfacial film protein rate). From chromatographic analysis it was shown that these aggregated moieties are mainly composed of the gum glycoproteins (small molecular weight), one of the Arabinogalactan-peptide population, and only involve the larger macromolecules (gum conjugates AGP) at large surface concentrations. Small angle neutron and X-ray scattering measurements on the species recovered from the interface, shed light on differences in their structure at both small and large scales, compared to native gum Arabic. Contrast match conditions were used to directly probe interfacial layers in macroscopic emulsions through SANS. The structure at large scales was shown to match that of gum Arabic in solution at a given concentration, while large differences were observed at lower scale, which was ascribed to the disordering of the native structures.

These three studies can now be used to propose some rational design of the formulation of gum Arabic stabilized emulsions. Gum Arabic is widely used in the food industry, in particular for the stabilization of beverage emulsions. These emulsions are stored as a concentrate that is diluted in the final product. Both concentrated and dilute emulsions need to remain metastable for several months or years in order to meet industrial specifications.

Since gum Arabic only contains a limited amount of surface-active species, it is often used in large excess in beverages formulation (>20w/w%). This results in an important concentration of non-adsorbed species in the aqueous phase, which may cause unwanted effects such as depletion, osmotic stress, pH and viscosity changes. However, high concentration of gum Arabic also corresponds to a high ionic strength, which promotes adsorption. We have demonstrated that an accurate control of physico-chemical parameters such as pH and ionic strength was an efficient lever to enhance the surface coverage of emulsion droplets, which may be used to counterbalance a concentration decrease. Indeed, increasing the surface coverage goes along with the formation of an extensive network at the oil/water interface, and emulsion metastability. A rational formulation design would be to decrease the amount of gum Arabic and increase salt concentration or decrease pH. Importantly, once the emulsion is formed, adsorbed layers are extremely resistant to further changes and will not relax their conformation. This resilience can be utilized to subsequently modify concentration, pH and ionic strength, the value of these parameters being only critical during emulsification.

A proof-of-concept experiment was performed using a very low concentration of gum Arabic to stabilize hexadecane-in-water emulsions. One sample was homogenized without additional ingredients while in the other two, either the pH was decreased or sodium chloride was added. The three samples were compared with an emulsion stabilized with ten times more gum Arabic. Size distributions of the emulsions after one hour of aging are presented in Figure 15.

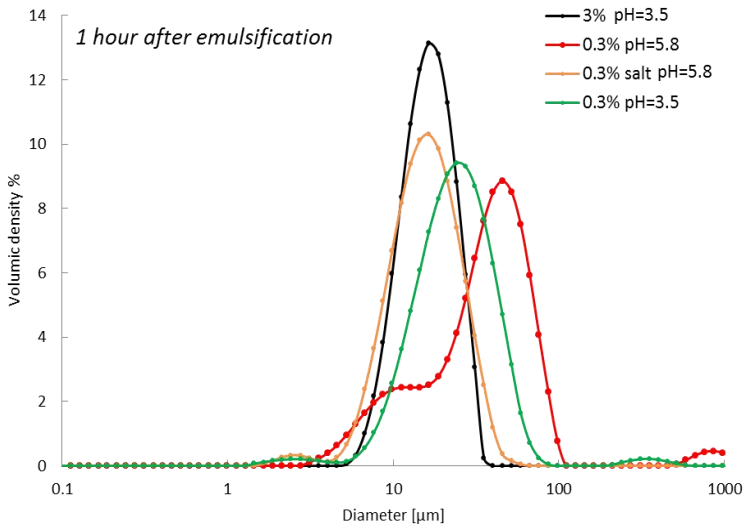

Figure 15: Size distribution of hexadecane-in-water emulsion (20v/v%) stabilized by gum Arabic one hour after emulsification with variations of physico-chemical parameters employed in the formulation (pH and salt concentration).

It is important to mention that right after emulsification all the emulsions displayed the same size distribution. After one hour, no noticeable change can be observed with the emulsion made with a solution concentrated in 3% w/w in gum at a pH of 3.5 (black curve). Dividing the gum concentration by 10 at same pH leads to a significant coarsening of the emulsion (green curve). Increasing the pH of the solution to 5.8 leads to a fast destabilization of the emulsion (red curve), while adding salt significantly compensate the pH increase, leading to a size distribution slightly unchanged compared to the initial state (orange curve). These primary observations further underline the importance of the physico-chemical parameters employed in a gum Arabic stabilize emulsion formulation.

Secondly hexadecane-in-water emulsions were stabilized using recovered species from the interface of previous gum Arabic stabilized emulsions. Two samples of recovered species were used: non aggregated or partially aggregated. Evolution of the emulsions size distributions after one hour and one week of aging are presented in Figure 16. Using the nitrogen rate of the sample of recovered species as compared to the nitrogen rate of gum Arabic, the concentration of stabilizer in the formulation was calculated to obtain an equi-nitrogen ratio between each formulation.

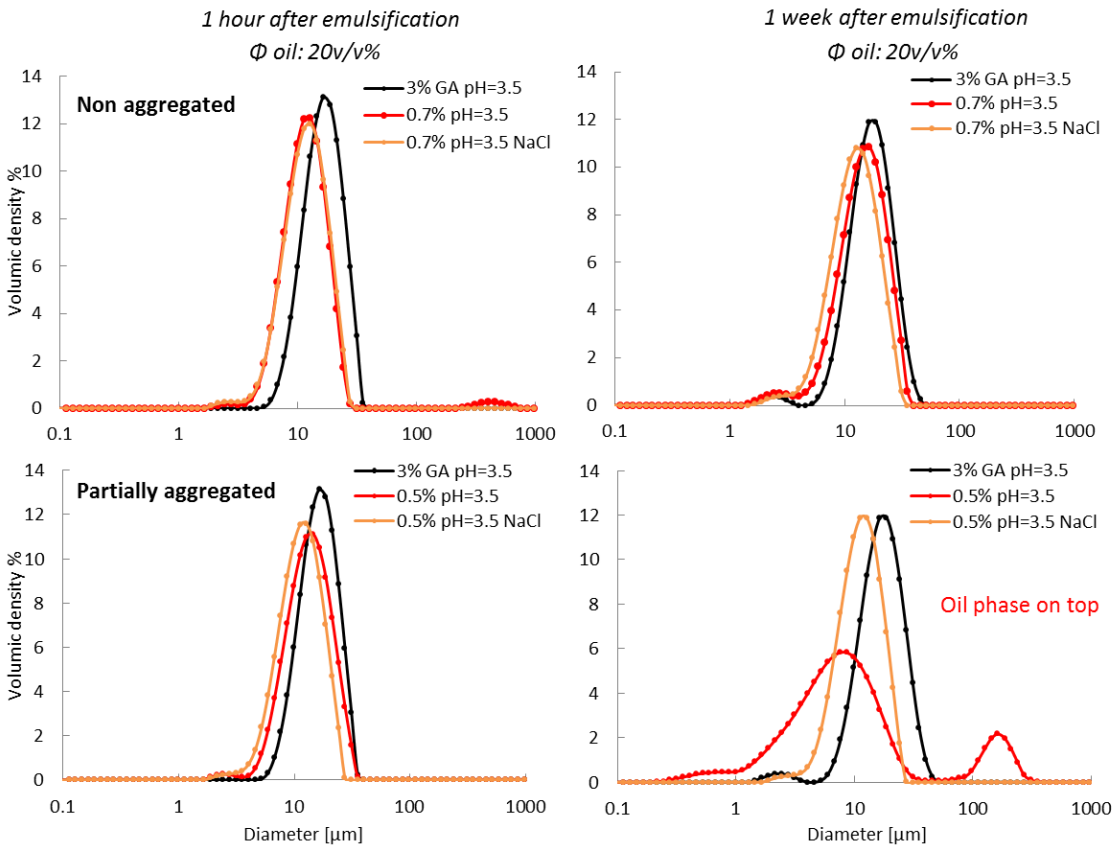

Figure 16: Time evolution of size distribution of hexadecane-in-water emulsion (20 v/v%) stabilized by species recovered from the interface. Effect of aggregation rate and salt (NaCl) concentration.

As observed from the size distributions, emulsions stabilized in these conditions gave slightly finer drops (orange and red curves) than when using native gum Arabic (black curve). After one week of aging, without addition of salt, oil-in-water emulsions formed with the non-aggregated recovered species remained quite metastable, whereas those formed with partially aggregated species destabilized quickly. This observation shows the importance of the interfacial structuration in the emulsion metastability. Indeed species irreversibly aggregated probably can no longer adsorb or provide interfacial aggregation, leading to a poor stabilization of droplets interface. Whereas using an addition of salt provided better emulsion metastability in both case, probably through an increased amount of interfacial agent adsorbed at the interface.

In a third stage a default of recovered non-aggregated species was used to stabilize a hexadecane-in-water emulsion. The evolution of the emulsion size distribution over time is presented in Figure 17.

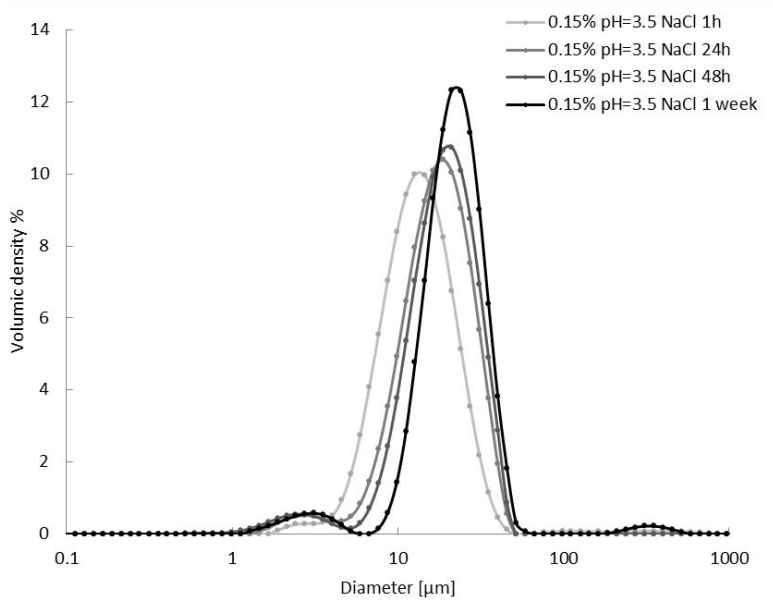

Figure 17: Evolution with time of the size distribution of a hexadecane-in-water emulsion stabilized by recovered species from the interface non-aggregated using a default of stabilizer.

Interestingly, over one week coalescence of the smaller droplets and a reduction of the emulsion polydispersity were observed. This behavior looks similar to that of Pickering emulsions which present a limited coalescence mechanism. It was previously shown in this manuscript that gum Arabic stabilized emulsion do not present such a mechanism, but it seems the most amphiphilic adsorbed species of gum Arabic favor limited coalescence events. This observation confirms that within the gum almost all the species have the ability to adsorb at oil/water interfaces, but only part of them possesses good stabilization properties, under the action of interfacial aggregation and steric repulsion. Therefore when using only the most surface active species of gum arabic to stabilize an emulsion, with a default of stabilizer regarding the available interfacial area, limited coalescence is a possible mechanism of stabilization.

Another observation pointed out in this thesis is the fact that emulsions that were forced to partially coalesce through centrifugation, possessed interfacial films with a higher protein rate and a more aggregated network. Hence, forcing emulsion droplets to undergo slight coalescence events might be a promising method to enhance emulsions metastability.

As a final conclusion, the detailed understanding of structures and phenomena at play in the emulsification and stabilizing properties of gum arabic shown in this thesis should prove itself to be a precious ally in the design of complex formulations.

Appendix: analytical techniques

The aim of this short appendix is to give a general overview of the different experimental techniques used in this thesis while specific experimental conditions are described in the materials and methods sections of each manuscript.

I- Emulsification

Emulsions were formed through mechanical dispersion. Micro-metric emulsions were dispersed with rotor/stator units (Ultra-turrax or Dispax). Emulsion droplets were sheared through a thin gap. In the case of the Dispax device, the sample was continuously recirculated as sheared. Both units gave emulsions droplets within the range 1-50 µm of mean diameters depending on the rotation speed and duration.

Figure A: Picture of an Ultra-turrax and a Dispax rotor/stator units.

II- Scattering

In this thesis we characterized objects of different dimension going from macromolecules to micro-metric droplets. The scattering source must be selected as a function of the object size. Light scattering is typically relevant for the micrometric domain, while X-ray and neutron small-angle scattering are useful in the nanometric domain.

i. Static light scattering

Static light scattering measurements were used to determine the size distributions and Sauter diameters also called the volume-surface diameter as described in equation (A.1) of gum Arabic stabilized oil-in-water emulsions. A monochromatic (633nm) laser beam is sent on a sample with object of refractive index differing from the dispersed media (usually water). Several detectors (at

different angle around the studied sample) collect the scattered beams. An analysis using the Mie theory is performed, which yields sample size volume distribution over a broad range of size.

$$d[3,2] = \frac{\sum_i n_i d_i^3}{\sum_i n_i d_i^2} \quad \text{(A.1)}$$

ii. Small angle neutron and x-ray scattering

Small angle scattering is a technique used to probe structures with characteristic size between 0.5 and 100nm. The experiment consists in sending a beam of neutron or X-rays with a controlled wavelength range onto a sample. A detector at a certain distance from the sample will detect the scattered intensity as a function of the angle of diffusion θ as shown in Figure B [1], [2].

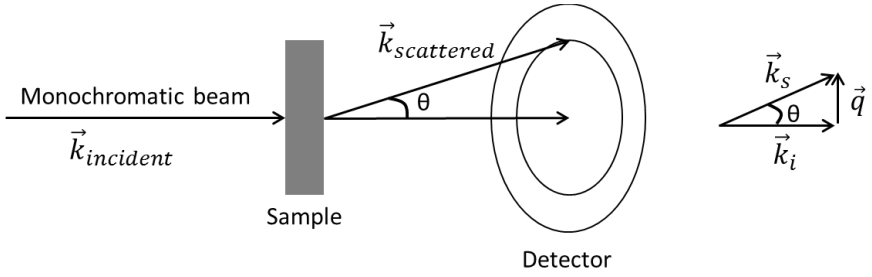

Figure B: Schematic representation of small angle scattering

The direction of the diffusion angle θ is called the scattering vector and is defined as:

$$\vec{q} = \vec{k}_{scattered} - \vec{k}_{incident} \quad \text{(A.2)}$$

The scattered wave is considered as elastic as we consider no exchange of energy between the neutrons and the sample as shown by equation:

$$\|\vec{k}_{scattered}\| = \|\vec{k}_{incident}\| \quad \text{(A.3)}$$

The magnitude of the scattering vector can be expressed by equation (A.4) where λ is the wavelength of the monochromatic beam (neutron or X-ray) and θ is the scattering angle as shown in Figure .

$$q = \|\vec{q}\| = \frac{4\pi}{\lambda}\sin\frac{\theta}{2} \quad \text{(A.4)}$$

The scattered intensity depends on shape, concentration and contrast of the scattering objects. The origin of contrast depends on the beam source used: a difference of electronic density for X-ray scattering and nuclei for neutron scattering. In neutron scattering, the contrast can be easily tuned through the use of deuterated compounds such as deuterated water. Neutron scattering is a powerful tool because contrast variation can be used to probe the structure of adsorbed species at

oil droplet interfaces as schematized in Figure C. Neutrons wavelengths are ranging between 0.2 and 2 nm while the X-ray wavelengths are around 0.1-0.2nm.

It is often convenient to decompose the scattered intensity in two functions which depend on the physical properties of the sample:

$$I(q) = N.V.\Delta\rho^2.P(q).S(q) \quad \text{(A.5)}$$

with N the number of particles, V the volume of a scatterer, Δρ the contrast parameter, P(q) the form factor (intra-objects correlations) and S(q) the structure factor (inter-objects correlations).

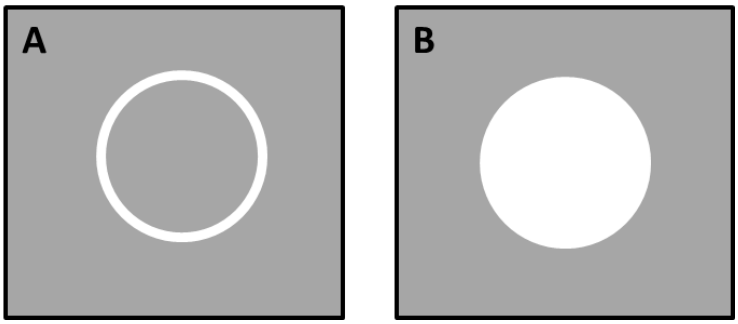

Figure C: Schematic representation of neutronic contrast variations for small angle scattering experiments on emulsion droplets (A) contrast is matched between the continuous and the dispersed phase to probe only the adsorbed film, (B) contrast is not matched between both phases. (Usually the dispersed phase is deuterated water).

Scattering spectra are presented as a function of the scattering vector q. The structural information is then averaged and displayed in the reciprocal space. High q values will deals with small length scales and low q values with larger length scales. A Kratky representation is often used to interpret scattering spectra of ordered/disordered proteins: it consists of representing the scattered intensity multiplied by the square of the scattering vector q as a function of q, $I(q).q^2 = f(q)$. The area under this curve is a scattering invariant, which is directly proportional to the total volume of scattering material. An evaluation of the radius of gyration can be directly inferred from peak positions in this representation [3].

Another general scattering theorem is the Porod law, which states that for smooth interfaces the intensity decays as q^{-4}. Other power laws are routinely observed corresponding to form factors of low dimension objects such as cylinders or disks (q^{-1}, q^{-2}) or structure factors of fractal networks (typically from $q^{-1.5}$ to $q^{-3.5}$). Polymer networks typically fall in this last category where the q^{-2} decay is the signature of random (Gaussian) coils [3].

III- Electrophoretic mobility

Electrophoretic mobility measurements were performed using a Zetasizer NanoZS instrument (Malvern Instrument) in DTS1060 cells.

Electrophoretic mobility μ_e of a colloidal solution is determined by measuring the velocity of the dispersed colloids under a uniform electric field as shown in Figure D. The counter-ions surrounding the particles will move in an opposite direction and thus generate a retardation force. μ_e is defined by equation (A.6) with v the drift velocity of the particles and E the electric field strength [4], [5].

$$\mu_e = \frac{v}{E} \tag{A.6}$$

The electrophoretic mobility is expressed in [µm.cm.V^{-1}.s^{-1}]. The Smoluchowski theory relates the electrophoretic mobility μ_e to the zeta potential ζ through the equation (A.7) with ε_r the dielectric constant of the dispersed medium, ε_0 the free space permittivity and η the dynamic viscosity of the dispersed medium:

$$\mu_e = \frac{\varepsilon_r \varepsilon_0 \zeta}{\eta} \tag{A.7}$$

However the Smoluchowski theory is only valid for colloids with a thin double layer (particle much larger than the Debye length).

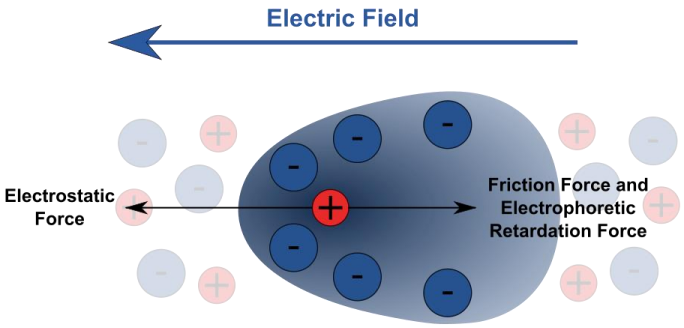

Figure D: Schematic representation of a positively charge colloidal particle (with its counter-ion cloud) moving in an electric field E.

Measurements on gum Arabic electrophoretic mobility were performed using a low concentration of gum. For higher gum concentrations the conductivity of the solution was two strong to be supported by the electrophoresis cells (electrodes were burned out).

IV- Interfacial tension

Interfacial tension measurements were carried out using the pendant drop method with a drop shape analyzer (Kruss ®). A drop of aqueous solution of gum Arabic was formed in a cell filled with hexadecane. Interfacial tensions were determined from the analysis of interface contour and the Young-Laplace equation, with σ the interfacial tension, R_1 and R_2 the principal radii of curvature and ΔP the pressure difference between both phases [6]:

$$\Delta P = \sigma\left(\frac{1}{R_1}+\frac{1}{R_2}\right) = \Delta P_0 - \Delta\rho g z \qquad \text{(A.8)}$$

ΔP_0 is the pressure difference at z=0 and Δρ is the density difference between the dispersed and continuous phase.

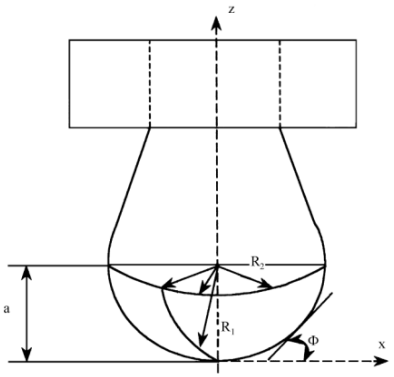

Figure E: scheme of a pendant drop

V- Chromatography

i. Size exclusion chromatography

Size exclusion chromatography is a separation technic based on the relative size or hydrodynamic volume of macromolecules. It takes advantage of the difference of retention time of macromolecules when they flow through a porous media. The chromatographic column being filled with porous beads, the size of the pore will determine the molar mass range it will separate. The largest macromolecules will not be retained within the pores whereas the smallest macromolecules will be retained longer inside the pores (Figure F).

Size exclusion columns can be calibrated for a separation condition in order to determine the statistical average molecular weight of a polymer as well as the molecular weight distribution. On-line molecular weight measurement needs adequate detectors such as: light scattering detector or a viscometer with universal calibration. [7]

A 7.8 mm x 300 mm BioSuite 450Å SEC column packed with 8µm silica beads was implemented. The average pore size of the silica beads was of 450 Å. The column was calibrated using branched dextran standards. [8]–[10]

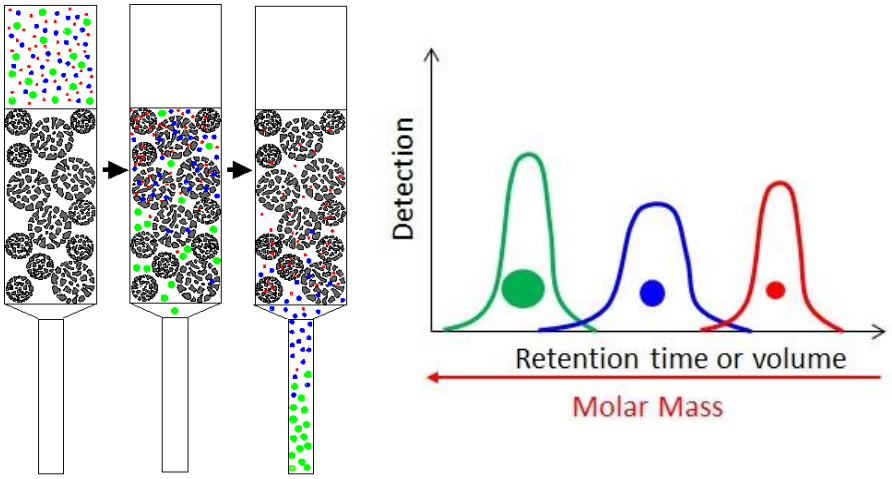

Figure F: Schematic representation of size exclusion chromatographic separation and detection

ii. Hydrophobic interaction chromatography

Hydrophobic interaction chromatography separates the molecules based on their hydrophobic properties. The mixture to separate is injected in the column under high salinity conditions. The high salt concentration reduces the solvation of the hydrophobic moieties, easing the adsorption of these on the hydrophobic ligands of the stationary phase. Then the salt concentration is gradually reduced along the separation which leads to the gradual desorption of the hydrophobic species [11]. Therefore the less hydrophobic species are eluted first whereas the more hydrophobic species are the last eluted, as illustrated in Figure . A 7.5 mm x 75 mm Biosuite Phenyl column packed with 10 µm silica beads coated with phenyl moieties was used. The average pore size of the silica beads was of 1000 Å to accommodate macromolecules with high molecular weight.

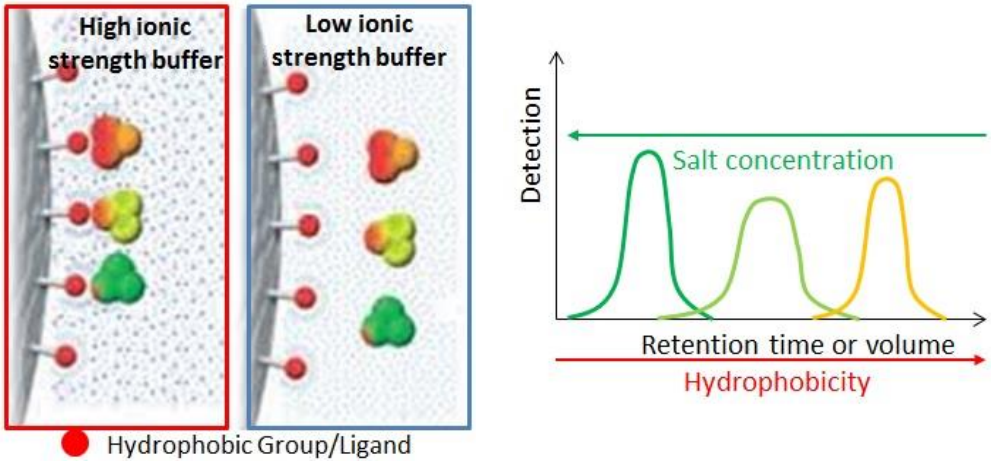

Figure G: Schematic representation of hydrophobic interaction chromatographic separation and detection

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Résumé

La gomme arabique, un exsudat d'arbre, est utilisée essentiellement pour ses propriétés stabilisantes et émulsifiantes. Ce produit naturel est un mélange complexe de protéines et de polysaccharides liés de façons covalentes qui différent dans leurs masse molaire et leurs hydrophobicités. Malgré le grand nombre d'études existantes dans la littérature sur la structure et les propriétés interfaciales de la gomme arabique, la compréhension de la relation entre la composition interfaciale, la structuration interfaciale, et la métastabilité d'émulsions reste compliquée. Dans la littérature la gomme est décrite comme un mélange de trois fractions : une fraction riche en polysaccharides (arabinogalactan), une fraction composée de conjugués protéine-polysaccharide et une fraction de glycoprotéines. La fraction de conjugués est identifiée comme responsable des propriétés émulsifiantes et stabilisantes de la gomme (des émulsions huile-dans-eau), avec la partie protéinée s'adsorbant à la surface des gouttes d'huiles et les espèces carbonées assurant de fortes répulsions stériques entre gouttes.

Dans cette thèse, nous avons étudié le comportement microscopique des espèces de la gomme en solution et aux interfaces huile/eau. La première étape a été de caractériser la structure des molécules de la gomme en solution. Une séparation chromatographique bi-dimensionnelle de la gomme a été réalisée par exclusion stérique suivie pas une séparation par affinité hydrophobe, permettant ainsi de confirmer la nature très hétérogène de la gomme. L'interprétation de spectres de diffusion de rayons-X et de neutrons aux petits angles obtenus avec des solutions de gomme arabique et de ses différentes fractions, nous a conduits à proposer un modèle structural de la gomme arabique en solution.

Puis nous avons étudié la composition de films interfaciaux pour des émulsions d'huile dans des solution de gomme arabique et comparé celle-ci avec la composition de la gomme en solution. Une augmentation du taux de protéine dans le film interfacial par rapport à la solution, a confirmé le rôle crucial des espèces polypeptidiques sur l'adsorption. Nous avons observé que la composition du film adsorbé diffère de celle de la gomme en solution, mais reste hétérogène en taille et en hydrophobicité. Un bilan massique a révélé une forte dépendance entre la formulation de l'émulsion (concentration en gomme, pH, salinité) et la concentration surfacique, sans changements significatifs de la composition interfaciale. Ces résultats suggèrent un changement de conformation des espèces adsorbées en fonction des conditions d'émulsification.

Dans un troisième temps, nous nous sommes intéressés aux mécanismes de stabilisation provenant de l'adsorption des espèces amphiphiles de la gomme arabique. Pour cela, nous avons développé une méthode permettant de récupérer les espèces adsorbées dans une émulsion huile dans eau. Cette méthode nous a permis de mettre à jour une structuration du film adsorbé. Les espèces récupérées à l'interface ont démontré l'existence d'un mécanisme d'agrégation, dont l'intensité dépendait directement du taux de couverture et de protéine de l'interface. La métastabilité d'émulsions stabilisées par de la gomme arabique a augmenté en promouvant la structuration interfaciale, c'est à dire en favorisant le taux d'agrégation des espèces adsorbées. Un tel comportement n'a pas encore été reporté dans la littérature et nous pensons qu'il s'agit d'un mécanisme clef dans le cadre d'émulsions stabilisées par la gomme arabique. Enfin, des expériences de diffusion de neutrons aux petits angles (contraste identique entre les phases continues et dispersées) ont révélé des différences de structuration entre deux régimes de concentration surfacique. Ces observations ont été discutées grâce à la comparaison des spectres de diffusion de la gomme en solution. Pour résumé, ce travail de thèse porte sur la relation entre composition et structuration dans des émulsions stabilisées par la gomme arabique, et démontre notamment l'existence d'un mécanisme original, probablement responsable de l'étonnante stabilité de ces émulsions.

Abstract

Gum arabic, a tree exudate, is essentially used for its binding and emulsifying properties. This natural product is a complex mixture of covalently linked proteins and charged polysaccharides moieties, differing in their molecular mass and hydrophobicity. A large body of literature now exists on the structure and interfacial properties of gum Arabic but a comprehensive description of the relationship between interfacial composition, interfacial structuration and emulsion metastability remains elusive. In the literature, gum Arabic is described as a mixture of three fractions: an arabinogalactan rich polysaccharide fraction, a polysaccharide-protein conjugates fraction and a fraction of glycoproteins. The conjugate fraction is thought to be responsible for the emulsifying and stabilizing properties of the gum, with the protein part adsorbing at oil droplets surface and the carbohydrate moieties providing steric repulsion between droplets.

In this work, we have investigated the microscopic behavior of the gum species in solution and at oil/water interfaces. The first step was to characterize the structure of gum Arabic species in solution. A two-dimensional separation of the gum molecules was performed using size exclusion chromatography followed with by hydrophobic interaction separation, which confirmed the highly heterogeneous composition of the gum. Small angle X-ray and neutron scattering measurements on the gum and its fractions led us to propose a structural representation of the gum conjugated moieties.

Then the composition of adsorbed gum Arabic films as compared to gum Arabic solutions has been investigated. An increase in the protein rate of the interfacial film showed the crucial role of the polypeptide moieties on the adsorption. The composition of the adsorbed film was shown to differ from the bulk but remained heterogeneous in size and hydrophobicity. A mass balance revealed a strong dependence between the emulsion formulation (gum concentration and physico-chemical parameters) and the surface concentration, while the composition of the interface was only slightly changed. These results suggest that gum Arabic adsorbing species must adopt conformational changes depending on emulsification conditions.

In a third stage, we have addressed the stabilization mechanisms resulting from the adsorption of gum Arabic amphiphilic species. For that purpose, we have developed a method to recover the adsorbed species within an oil-in-water emulsion. This method allowed us to unveil a structuration of the adsorbed film. Species recovered from the interface displayed aggregation, the magnitude of which directly depended on the coverage density and protein rate of the adsorbed film. The metastability of emulsions, stabilized with gum Arabic, increased upon promoting interfacial structuration, i.e. when the aggregation rate of adsorbed species was enhanced. Such behavior has not been reported so far in the literature and we believe that it is a key mechanism of gum Arabic-based on emulsions. Finally, small angle neutron scattering experiments (contrast match between the continuous and dispersed phases) disclosed differences of structuration between two regimes of interface coverage. These observations were discussed in the light of the comparison with the scattering spectra of gum Arabic solutions. To conclude, this thesis revolves around the composition/structuration relationship in gum Arabic-stabilized emulsion stabilized and demonstrates that an original mechanism is at play in this complex system.